Lund 1976

STUDIES ON THE PREPARATION OF
RAPESEED PROTEIN ISOLATES

LARS GILLBERG
CIV. ING., GB

DEPARTMENT
OF
CHEMICAL ENGINEERING

AVDELNINGEN FÖR KEMISK TEKNOLOGI

CHEMICAL CENTER
LUND INSTITUTE OF TECHNOLOGY — UNIVERSITY OF LUND
LUND-SWEDEN

STUDIES ON THE PREPARATION OF RAPESEED PROTEIN ISOLATES

LARS GILLBERG

CIV. ING., GB

AKADEMISK AVHANDLING SOM FÖR AVLÄGGANDE AV TEKNISK DOKTORS-EXAMEN OFFENTLIGEN KOMMER ATT FÖRSVARAS I HÖRSAL C, KEMI-CENTRUM, LUND, TISDAGEN DEN 25 MAJ 1976, KL 13.15.

STUDIES ON THE PREPARATION OF RAPESEED PROTEIN ISOLATES

LARS GILLBERG

LUND 1976

STUDIES ON THE PREPARATION OF RAPESEED PROTEIN ISOLATES

Lars Gillberg, Department of Chemical Technology, Chemical Center, The Lund Institute of Technology, P.O. Box 740, S - 220 07 LUND 7, Sweden

This thesis is a summary of the results from the following papers, referred to in the text by their Roman numerals:

I Gillberg, L. and Törnell, B. 1976. Preparation of rapeseed protein isolates I. Dissolution and precipitation behaviour of rapeseed proteins. J. Food Sci. In press.

II Gillberg, L. and Törnell, B. 1976. Preparation of rapeseed protein isolates II. Precipitation of rapeseed proteins in the presence of polyacids. J. Food Sci. In press.

III Lönnerdal, B., Gillberg, L. and Törnell, B. 1976. Preparation of rapeseed protein isolates III. A study of rapeseed protein isolates by molecular sieve chromatography. Submitted for publication.

IV Åman, P. and Gillberg, L. 1976. Preparation of rapeseed protein isolates IV. A study of the distribution of carbohydrates in the preparation of rapeseed protein isolates.

STUDIES ON THE BIOSYNTHESIS OF WHEYSEED PROTEIN ISOLATES

Lara Gilliver, Department of Chemical Technology, Chemical Centre, The Lord Institute of Technology, P.O. Box 1740, S - 220 07 LUND S. Sweden

This thesis is a summary of the results from the following papers, referred to in the text by using Roman numerals:

I Gilliver, L. and Lindahl, B. 1974. Preparation of rapeseed protein isolates I. Dissociation and precipitation behaviour of rapeseed proteins. J. Food Sci. In press.

II Gilliver, L. and Lindahl, B. 1974. Preparation of rapeseed protein isolates II. Precipitation of rapeseed proteins in the presence of polyanions. J. Food Sci. In press.

III Lönnerdal, B., Gilliver, L. and Lindahl, B. 1974. Preparation of rapeseed protein isolates III. A study of rapeseed protein isolates by polyacrylamide chromatography. Submitted for publication.

IV Man, P. and Gilliver, L. 1975. Preparation of rapeseed protein isolates IV. A study of the distribution of carbohydrates in the preparation of rapeseed protein isolates.

Introduction

Rapeseed ranks fifth among the oilseed crops in the world and is the dominating one grown in Sweden. The seed contains about 40% oil and 25% protein. The rapeseed protein has a very well balanced amino acid composition and is, compared to other oil seed proteins, rich in the sulphur containing amino acids. These facts make rapeseed attractive as a raw material for the manufacture of food protein products such as protein isolates.

Protein isolates are usually prepared by first dispersing the raw material in an aqueous alkaline solution. After removal of the insoluble material the dissolved proteins are recovered by acidification. The precipitate, which is commonly washed and dried before use, constitutes the protein isolate.

The dissolution of nitrogen- and phosphorus-containing compounds from rapeseeed meal

In introductory experiments defatted rapeseed meal was treated with aqueous solutions of various pH. The slurries were centrifuged and the supernatants were analyzed for nitrogen. In these experiments it was observed that more or less pronounced white bands appeared in the sediments. Analysis of such bands showed that they contained high amounts of mineral salts (mainly Mg, Ca, K and Na) of phytic acid. The presence of phytic acid in a food product is known to have an adverse effect on the nutritive value of the product. It can exert this effect by forming complexes with the proteins, which may obstruct the enzymatic

Introduction

Rapeseed ranks fifth among the oilseed crops in the world and is the dominating one grown in Sweden. The seed contains about 40% oil and 25% protein. The rapeseed protein has a very well balanced amino acid composition and is, compared to other oil seed proteins, rich in the sulphur containing amino acids. These facts make rapeseed attractive as a raw material for the manufacture of food protein products such as protein isolates.

Protein isolates are usually prepared by first dispersing the raw material in an aqueous alkaline solution. After removal of the insoluble material the dissolved proteins are recovered by acidification. The precipitate, which is commonly washed and dried before use, constitutes the protein isolate.

The dissolution of nitrogen and phosphorus-containing compounds from rapeseed meal

In introductory experiments defatted rapeseed meal was treated with aqueous solutions at various pH. The mixtures were centrifuged and the supernatants were analyzed for nitrogen. In these experiments it was observed that more or less pronounced white bands appeared in the sediments. Analysis of such bands showed that they contained high amounts of mineral salts (mainly Mg, Ca, K and Na) of phytic acid. The presence of phytic acid in a food product is known to have an adverse effect on the nutritive value of the product. It can exert this effect by forming complexes with the proteins, which may obstruct the enzymatic

digestion of the protein (Barré, 1956) and by forming insoluble salts in the intestines with calcium, magnesium, copper, zinc, manganese and iron in the food thus obstructing the adsorption of these important minerals (cf. e.g. Laurent, 1955; Taylor, 1965; Reinhold et al., 1973). It was also observed that rapeseed protein isolates rich in phytic acid and containing calcium or magnesium showed poorer resolubility and a lower swelling than isolates with a low phytic acid content. Thus, from many points of view, it was of interest to study possible ways of producing rapeseed protein isolates free from phytic acid.

For this reason a rather extensive study of the influence of pH on the dissolution of nitrogen, total phosphorus and phytic acid from defatted rapeseed meal was carried out. The nitrogen solubility curve had two minima. Even at the lowest of these minima (pH 4) the nitrogen solubility was rather high (40%). This behaviour is probably a consequence of the complicated protein composition of rapeseed, which covers proteins with widely different isoelectric points and molecular weights. Thus, a large proportion of the proteins is characterized by an isoelectric point close to 11 and a molecular weight of about 13 000 (Lönnerdal and Janson, 1972). The other proteins have isoelectric points spread out in the interval from pH 4 to 8 and can, with respect to their molecular weights, be divided into three groups with molecular weights of about 75 000, 150 000 and 320 000 (Lönnerdal, 1976). The solubility of phytic acid in rapeseed meal was found to vary with pH in a complex manner. It could be explained, however, by considering the fact

that phytic acid can react with metal ions present in the meal as well as with the proteins. It was found that the solubility of phytic acid could be supressed by the addition of calcium or magnesium salts (I).

From the solubility of the total phosphorus and of phytic acid, the solubility of the non-phytic phosphorus in rapeseed meal was calculated. This calculation showed that the solubility of the non-phytic phosphorus varied with pH in a very complicated way. It was suggested that the non-phytic phosphorus to a large extent originated from the presence of nucleic acids and that the complex solubility behaviour of the non-phytic phosphorus was due to complex formation between nucleic acids and proteins with different isoelectric points (I), a hypothesis which has not yet been tested.

The fact that protein products from rapeseed have not been used for food purposes is mainly due to the high content of glucosinolates in the seed. The glucosinolates are water soluble substances, which under the influence of enzymes (myrosinase) present in the seed can be hydrolyzed to give water soluble as well as oil soluble substances, some of which have a goitre action. These compounds have also a deleterious effect on the oil quality and can cause poisoning of the catalyst used in the hydrogenation of the rapeseed oil. Thus, from many points of view, it is important to prevent the hydrolytic decomposition of the glucosinolates. One way of doing this is to inactivate the myrosinase by subjecting the seeds to a heat treatment. Such a

treatment, however, may decrease the solubility of the proteins. Experiments were carried out to study the influence of a heat treatment on the nitrogen and phytic acid solubility. As expected, the nitrogen solubility decreased by a heat treatment of the seeds. However, at alkaline pH:s the decrease in nitrogen solubility was found to be relatively small. For the mildest heat treatment tested, which according to Appelqvist and Josefsson (1967) should be sufficient to give an almost total myrosinase inactivation, the nitrogen solubility at pH 11 was hardly affected. The solubility of nitrogen, total-, phytic- and non-phytic phosphorus was found to vary with pH in almost the same way with meal from heat-treated seeds as with meal from non-heat-treated seeds. The dissolution studies showed that it was possible to produce a rapeseed meal from heat-treated seeds, possessing a high nitrogen solubility and a low phytic acid solubility at pH:s around 11 (I). For the preparation of rapeseed protein isolates in a high yield and with a low content of phytic acid, this pH-range would thus be appropriate for the extraction of the meal. As expected, phytic acid was found to be accumulated in the meal residue when rapeseed meal was extracted at pH 11.1. Analysis showed that the meal residue contained, calculated on its dry matter content, 5.2% phytic acid, 4.9% nitrogen, 21.9% neutral sugars and 12.0% Klason-lignin. Most of the Klason-lignin, probably derived from polyphenolic compounds present in the seed coats (Theander et al., 1976). Sugar analysis indicated that the dominating polysaccharides in the meal residue were cellulose, amyloids and arabinogalactans (IV).

Precipitation of rapeseed proteins by acidification

A rapeseed meal extract prepared by extracting rapeseed meal at pH 11.1 was, by molecular sieve chromatography, shown to contain four protein fractions with molecular weights of about 13 000, 50-75 000, 150 000 and over 250 000. When the pH of the extract was adjusted to 6.6 all proteins with molecular weights of 150 000 and higher were precipitated. The precipitate (from here on denoted Isolate A) was rich in acidic proteins. This result is in agreement with the fact that the high molecular weight rapeseed proteins have low isoelectric points (Ohlson and Sepp, 1975). Other proteins in the meal extract were also found to be present in Isolate A, which contained proteins with a wide range of isoelectric points (III).

The amino acid composition of Isolate A was very well balanced for human needs. It had a chemical score of 100, calculated with the reference amino acid pattern suggested by FAO 1973 (III).

Although acidification of the alkaline rapeseed meal extract resulted in a quantitative recovery of the high molecular weight proteins, less than 50% of the total nitrogen content of the meal extract was recovered in the precipitate. A large amount of the proteins in the meal extract thus remained in solution. This result is in agreement with previous studies on the preparation of rapeseed protein isolates (Pokorny et al., 1963; Shaikh et al., 1968; Sosulski and Bakal, 1969; Gillberg and Törnell, 1970; Kodagoda et al., 1973; Girault, 1973). The curve of the nitrogen recovery versus the pH of precipitation

was found to have a broad maximum and it was found that only a slight increase in the nitrogen recovery was obtained by carrying out the acidification in two steps (I).

When comparing the precipitation behaviour of a rapeseed meal extract prepared from a meal from heat-treated seeds with that from non-heat-treated seeds, the main difference consisted in a displacement by about 0.5 to 1 pH unit of the nitrogen precipitation curve towards higher pH values. It was also found that the buffer capacity of rapeseed meal to sodium hydroxide was lower if the seeds had been subjected to a heat treatment (I). Both these results reflect an influence of the heat treatment on the apparent basicity of the proteins similar to that observed in meat, where it has been shown that a heat treatment results in an uncovering of histidine groups, making the protein more basic (Conell and Howgate, 1964; Hamm, 1965).

The protein content of Isolate A as determined on an oven dry basis was calculated as 83.6% (N x 5.5) and, as expected, no phytic acid could be detected in the isolate. The glucosinolate content of the isolate was found to be very low and the content of neutral sugars was determined as 2.8% of which 34.8% was found to be ribose. The ribose was assumed to derive from RNA. From the ribose content the RNA content of the isolate was calculated to be 2.1%, assuming that the four RNA bases were present in equal amounts. Excluding the ribose the content of neutral sugars in the isolate was calculated as 1.8%. Acidic sugars were also shown to be present in the isolate. The total carbohydrate content of the isolate therefore is larger than 1.8% (IV).

As suggested by the influence of pH on the dissolution of phytic acid from rapeseed meal, a small change in the pH of extraction would have a large effect on the phytic acid content of the isolate prepared from the extract. This is illustrated by the following example. A protein isolate was prepared by extracting a meal from non-heat-treated rapeseeds at pH 10.2 and precipitating the isolate at pH 8.4. This isolate contained 11.5% phytic acid (Törnell et al., 1972), whereas Isolate A, prepared by extraction at pH 11.1 and precipitation at pH 6.6, contained no detectable amount of phytic acid (IV).

The presence of phytic acid in the rapeseed meal extract was shown to result in a considerable increase in the nitrogen recovery and also to bring about a remarkably pronounced increase in the dry matter content of newly separated isolates. Thus, it was found possible to prepare protein isolates with dry matter contents of up to about 50% by a careful choice of extraction and precipitation pH:s, and the nitrogen recovery could be increased from 34 to 78% by the addition of sodium phytate to a meal extract prior to its acidification (I). The precipitation pH giving a maximum in nitrogen recovery was found to coincide with or to occur at a lower pH than that giving a maximum in the dry matter content of the newly separated precipitates.

Experiments were carried out in order to find other substances having the same beneficial effect on the dry matter content and nitrogen recovery as phytic acid whilst not possessing its ad-

verse effect on the nutritive value of the food product. Some work along this line is presented in the next section.

Precipitation of rapeseed proteins in the presence of polyacids

The protein isolates discussed in this section were prepared from a meal extract obtained by extraction of rapeseed meal at pH 11.1. To the extract was added a polymeric anion and the isolates were precipitated by acidification. Anionic polymers used were sodium hexametaphosphate (HMP), carboxymethylcellulose (CMC), alginate, polygalactouronic acid as well as kappa-, iota- and lambda-carrageenans. All of these polymers were shown to be able to produce a very pronounced increase in the nitrogen recovery and, except for the carrageenans, to bring about a considerable increase in the dry matter content of the newly separated isolates. The nitrogen recovery, the dry matter and nitrogen contents of the isolates were shown to vary with the type and amount of polymer added, the pH of precipitation and the ionic composition of the extract. The maximum in the nitrogen recovery was found to occur at a pH value somewhat lower than that giving a maximum in the dry matter content of the precipitate (II).

Several results indicated that the effect of the added polyanion on the nitrogen recovery was a consequence of the formation of insoluble complexes between proteins and polyanions. These reactions were supposed mainly to be due to electrostatic interactions between cationic amino acid residues on the

proteins and anionic groups on the added polymers. Thus, in experiments with CMC it was found that the larger the amount of CMC added, the lower was the pH for maximum nitrogen recovery. In experiments where HMP was added to a meal extract, 0.08 g HMP/g protein and protein isolates prepared from aliquots of this mixture, it was found that a decrease in the pH of precipitation increased the P/N-ratio of the precipitates almost linearly. At pH 6.0 the P/N-ratio in the precipitate was 0.045 and at pH 3.0 it was 0.063 moles/mole. When compared on a weight basis, anionic polymers with a large charge density were found to precipitate proteins more efficiently than those with a low charge density. Thus, CMC with a high DS (DS = Degree of Substitution; the average number of carboxyl groups per anhydroglucose unit) precipitated protein more efficiently than CMC with a low DS. Among the carrageenans lambda-carrageenan, which had the highest content of sulphate groups, was found to be the most efficient precipitation aid.

The formation of insoluble complexes between polyanions and proteins has previously been observed in numerous systems (Bungenberg de Jong, 1949; Morawetz and Hughes, 1952; Nitschmann et al., 1959; Hidalgo and Hansen, 1969; Spinelli and Koury, 1970).

As expected, the electrolyte composition of the meal extract was found to have a large effect on the precipitation behaviour of the isolates. The addition of sodium chloride to a meal extract resulted in a decrease in the nitrogen recovery and dry matter content of the precipitates. Divalent ions (cations as

well as anions) had a greater effect than monovalent ions. In a separate study (Gillberg and Törnell, 1974) carried out with a rapeseed meal extract free from phytic acid, obtained by extraction of the meal at pH 8.2 in the presence of added $MgCl_2$, the amount of polymeric anion needed to obtain a maximal nitrogen recovery was considerably higher than the amount needed in similar experiments carried out in the absence of added salts as described in paper II.

In experiments where different amounts of CMC or HMP were added to an alkaline meal extract and the isolates precipitated by acidification, the nitrogen recovery went through a very pronounced maximum as the amount of CMC added was increased. At the highest concentration of CMC tested, the nitrogen recovery was much lower than that without addition of CMC. At additions of CMC beyond the maximum in nitrogen recovery the reactions between CMC and proteins obviously yielded negatively charged soluble complexes. This behaviour is expected from any high molecular weight polyacid. HMP, however, could be added in excess without producing a significant decrease in the nitrogen recovery. HMP, thus, was unable to bring about a charge reversal of the proteins and differed from CMC in this respect. This property of HMP is assumed to be a consequence of its low DP (DP = Degree of Polymerization; the number of monomeric units of which a polymer is built). Commercial HMP:s often have an average DP of about 10 - 20. The CMC samples used had average DP:s of about 700 - 1 500. Experiments showed that additions of HMP in low amounts to a rapeseed meal extract followed by aci-

dification and centrifugation gave supernatants with a low and almost constant phosphorus content, indicating that most of the added polyphosphate was bound in the precipitate. At larger additions of HMP the concentration of phosphorus in the supernatant increased and at the largest additions tested the increase of phosphorus in the supernatant corresponded closely to the increase in the amount of HMP added (II).

A study by molecular sieve chromatography showed that all extracted rapeseed proteins could be precipitated, either in a one-step or in a two-step process, using CMC or HMP as a precipitation aid. The study also showed that all added CMC could be recovered in the precipitate (III). Since all other polyanions tested were as effective as CMC and HMP in improving the nitrogen recovery (II), it is likely that most anionic polymers can be used for a quantitative precipitation of rapeseed proteins.

No ribose or uronic acids could be detected in the supernatant obtained after a quantitative precipitation of rapeseed proteins using HMP as a precipitation aid. Thus, all of the RNA and also all of the acidic rapeseed sugars in the meal extract were accumulated in the protein isolate (IV). This, most probably, was a consequence of the formation of insoluble complexes between the polyanions and the proteins at the pH of precipitation. There can be no doubt that in the formation of protein isolates from other raw materials interactions between proteins and polyanions present in the raw material, e.g. phytic acid,

RNA, acidic polysaccharides and acidic polyphenols are also most important.

When preparing the supernatant mentioned above, 54% of the neutral sugars of the meal were recovered in the meal residue and 43% in the supernatant. About 90% of the dissolved carbohydrates of the supernatant were found to be oligosaccharides, mainly sucrose, and the remaining part arabinans and arabinogalactans.

Acknowledgements

First I want to express my deep gratitude to Professor Bertil Törnell for initiating this work and for all his support during the course of the work.

Many thanks are also expressed to all my colleagues throughout the country for stimulating discussions, for experimental help and for giving me access to their laboratorial facilities. I specially want to thank Mrs Jana Polackova for technical assistance, Dr Bo Lönnerdal and Mr Per Åman for their most valuable cooperation, Dr Ragnar Ohlson, Dr Klas Anjou and Mr Klas Magnusson and other colleagues at AB Karlshamns Oljefabriker for their continuous interest, for the supply of raw material and for analytical and laboratorial help, Mr Sigvard Winsnes, Mr Sven-Eric Kroon, Dr Göran Krook and Dr Jan Teär for giving me access to the pilot plant of AB Alfa Laval, Dr Sten-Åke Liedén, Dr Anne-Marie Hermansson and Dr Roy Nilsson and his colleagues at the Swedish Meat Research Institute for their interest and valuable

cooperation, Mrs Gunilla Cederholm for typing the manuscripts and Mrs Eva Tjerneld and Mr Gert-Anders Carlsson for the preparation of the diagrams involved in this thesis.

This work was carried out on grants given from the Swedish Board for Technical Development. This support is gratefully acknowledged.

References

Appelqvist, L.-Å. and Josefsson, E. 1967. Method for quantitative determination of isothiocyanates and oxazolidinethiones in digests of seed meals of rape and turnip rape. J. Sci. Food Agr. 18(11):510.

Barré, R.M. 1956. Influence de l´acide phytique sur la digestion pepsique de différentes proteines. Ann. pharm. franc. 14:182.

Bungenberg de Jong, H.G. 1949. Complex colloid systems. In "Colloid science", Vol. 2, p. 335. Ed. Kruyt, H.R. Elsevier Publishing Company, The Netherlands.

Conell, J.J., Howgate, P.F. 1964. The hydrogen ion titration curves of native, heat-coagulated and frozen-stored myofibrils of cod and beef. J. Food Sci. 29:717.

Gillberg, L. and Törnell, B. 1970. "Framställning av spinnbart rapsproteinisolat". Föredrag vid proteindag, p. 70. Dept. of Food Technology, Chemical Center, Lund, Sweden.

Gillberg, L. and Törnell, B. 1974. Preparation of rapeseed protein isolates. Paper read at IV International Congress of Food Science and Technology, Madrid.

Girault, A. 1973. The study of some properties of rapeseed protein with a view to protein concentrate production. J. Sci. Fd. Agric. 24:509.

Hamm, R. 1965. Heating of muscle systems. In Eds. Briskey, E.J., Cassens, R.C., Trautman, J.G. The physiology and biochemistry of muscle as a food, p. 363. University of Wisconsin Press, Madison, USA.

Hidalgo, J., Hansen, P.M.T. 1969. Interaction between food stabilizers and β-lactoglobulin. J. Agric. Food Chem. 17(5):1089.

Kodagoda, L.P., Young, C.Y., Nakai, S., Powrie, W.D. 1973. Preparation of protein isolates from rapeseed flour. Can. Inst. Food Sci. Technol. J. 6(3):135.

Laurent, S.-O. 1955. Fytinsyrans och fytasenzymets betydelse i näringskemin. Finska Kemists. Medd. 64:12.

Lönnerdal, B., Janson, J.-C. 1972. Studies of Brassica seed proteins. I. The low molecular weight proteins in rapeseed. Isolation and characterization. Biochim. Biophys. Acta 278:175.

Lönnerdal, B. 1976. Private communication.

Morawetz, H., Hughes, W.L. Jr. 1952. The interaction of proteins with synthetic polyelectrolytes. I. Complexing of bovine serum albumin. J. Phys. Chem. 56:64.

Nitschmann, von Hs., Rickli, E., Kistler, P. 1959. "Ueber dem Einfluss der Polyphosphate auf die Löslichkeit einiger Plasmaproteine und die Möglichkeit der Plasma-Fraktionierung mit Polyphosphat". Helv. Chim. Acta 42:2198.

Ohlson, R., Sepp, 1975. "Proteins and other crucifers" in Ed. Pirie, N.W. Food protein sources, Cambridge University Press.

Pokorný, J., Vodička, M., Žalud, J. 1963. Rapeseed III. The precipitation of rapeseed proteins from alkaline solutions with diluted acids. Sci. Papers Inst. Techn. Prague, Food Techn. 7(2):167.

Reinhold, J.G., Nasr, K., Lahimgarzadeh, A., Heydayati, H. 1973. Effects of purified phytate and phytate rich bread upon metabolism of zinc, calcium, phosphorus, and nitrogen in man. The Lancet 10:283.

Shaikh, I.A., Arshad, M., Haque, M.Y., Ali, S.M. 1968. Nutritional properties of rapeseed protein concentrate prepared from commercial cake. Pakistan J. Sci. Ind. Res. 11:263.

Sosulski, F.W., Bakal, A. 1969. Isolated proteins from rapeseed, flax and sunflower meals. Can. Inst. Food Technol. J. 2(1):28.

Spinelli, J., Koury, B. 1970. Phosphate complexes of soluble fish proteins. Their formation and possible uses. J. Agr. Food Chem. 18(2):284.

Taylor, T.G. 1965. The availability of the calcium and phosphorus of plant materials for animals. Proc. Nutr. Soc. 24:105.

Theander, O., Åman, P., Miksche, G., Yasuda, S. 1976. Carbohydrates, lignin and polyphenols in turnip rapeseed and white mustard hulls of different colours. To be published.

Törnell, B., Gillberg, L., Larsson, L. 1972. Framställning av proteinisolat ur raps. Swedish Board for Technical Development. Report 70-1434/U 1074.

PREPARATION OF RAPESEED PROTEIN ISOLATES

I. Dissolution and precipitation behavior of rapeseed proteins.

Lars Gillberg and Bertil Törnell

The Lund Institute of Technology
Department of Chemical Technology
Chemical Center
P.O.B. 740
S - 220 07 Lund 7
Sweden

Rapeseed protein isolates.

PREPARATION OF RAPESEED PROTEIN ISOLATES. I. Dissolution and precipitation behavior of rapeseed proteins. L. Gillberg and B. Törnell. J. Food Sci.

The dissolution of nitrogen and phosphorus containing substances from defatted rapeseed, and the subsequent precipitation of these substances by acid was studied.

The dissolution of the substances of interest was found to vary in a complicated manner with the pH of extraction. This was especially true for the dissolution of inositol hexaphosphoric acid (phytic acid) and the other phosphorus containing substances.

The presence of phytic acid in the protein extracts strongly affected the nitrogen recovery, the useful pH range for precipitation and the dry matter content of the precipitates. These effects were different with extracts prepared at different pH values. With an extract prepared at pH 11.1, addition of sodium phytate increased the yield from about 35 to 75 per cent of the extracted nitrogen and the dry substance content of the precipitate from 16 to 33 per cent.

INTRODUCTION

Rapeseed ranks fifth among the world´s oilseeds and is the dominant one grown in Sweden. The seeds contain about 40% oil and 25% protein. The rapeseed protein has a very well balanced amino acid composition and is especially rich in sulphur containing amino acids. These facts explain the current interest in rapeseed as a new source for the manufacture of food quality protein products such as protein isolates.

A protein isolate is usually prepared by first dispersing the raw material in an aqueous alkaline solution. After removal of insoluble material, the dissolved proteins are recovered by acid precipitation. The precipitate, which is commonly washed and dried before use, constitutes the protein isolate. In this paper, which is the first in a series, results from a study of this type of process using defatted rapeseed as a raw material will be reported. As will be shown, a process of this type gives fairly low yields and produces wheys with a high protein content. This may be a consequence of the fact that rapeseed has a very complicated protein composition and contains proteins with widely different iso-electric points and molecular weights. Thus, a large proportion of the proteins, 20-40%, is characterized by an iso-electric point close to 11 and a molecular weight of about 13,000 (Lönnerdal and Jansson, 1972). The other proteins have iso-electric points spread out in the interval from pH 4 to 8 and can, with respect to their molecular weights, be divided into three groups with molecular weights of 320,000, 150,000 and 75,000 (Lönnerdal, 1975).

The fact that rapeseed proteins traditionally are not used for food purposes is mainly due to the high content of glucosinolates in rapeseed. Glucosinolate contents up to 7.77% have been reported (Josefsson and Appelqvist, 1968). The glucosinolates are water soluble substances, which, under the influence of enzymes (myrosinase) present in the seed, can be hydrolyzed to give water soluble as well as oil soluble substances with a goiter action. One way to prevent the hydrolytic decomposition of the glucosinolates, and hence the formation of toxic degradation products, is to inactivate the myrosinase by subjecting the seeds to a heat treatment. Such a treatment, however, may decrease the solubility of the proteins, and hence have a negative effect on the yield of the protein isolate. In order to elucidate this latter effect, experiments with rapeseeds subjected to different heat treatments have been included in this study.

Results from introductory experiments indicated that phosphorus containing substances present in the seed might under certain conditions play a very important role in the precipitation step. The effects observed to the largest part could be referred to an influence of phytic acid (inositol hexaphosphoric acid) or of phytates. These findings initiated a separate study of the dissolution of phosphorus containing substances from defatted rapeseeds and of the effect of phytic acid on the precipitation of the proteins by acid. This part of the study is of wider interest than purely technical since phytic acid is likely to affect also the nutritional value of the product. Thus, the presence of phytic acid in food, by way of complex formation with

protein and with di- and polyvalent metal ions, may obstruct the enzymatic degradation of the protein (Barré, 1956) and the adsorption of calcium, zinc and iron (Reinhold et al., 1973).

References to previous work on the preparation of rapeseed protein isolates using aqueous alkaline extraction can be made to Girault (1973), Kodagoda et al. (1973), Sosulski and Bakal (1969), Shaikh et al. (1968), and to Pokorny et al. (1963). A method for the preparation of rapeseed protein isolates using extraction with 1 M NaCl has been described by Owen et al.(1971). Those works have largely treated the yield and nutritive value of the isolates. No previous work seems to have been published on the influence of pH on the extractability of the nitrogen and phosphorus containing compounds in rapeseed meal or on how the yield and properties of rapeseed protein isolates are influenced by phytic acid.

MATERIALS AND METHODS

Preparation of rapeseed meal

Rapeseed (*Brassica napus*, winter variety Panter) was obtained from AB Karlshamns Oljefabriker, Sweden.

Rapeseed meal of type A: About 5 kg of rapeseeds were crushed on a roller mill and the seed hulls removed by wind sifting. The oil from the dehulled seed fraction was extracted by hexane in a Soxhlet apparatus and the extracted flakes desolventized without heating. The dehulled, defatted flakes were disintegrated in a hammer mill (Kamas SKB 200). The product obtained

had a water content of 7.2% and contained (as calculated on an oven dry basis) 8.4% nitrogen and 1.3% phosphorus, 80% of which was shown to be derived from phytic acid.

Rapeseed meal of type B: About 5 kg of rapeseeds were conditioned at room temperature to a water content of 8% and were subjected to a heat treatment in an externally heated rotating drum for 18 minutes at 90 ^{o}C. The seeds were then crushed, extracted and desolventized as described above. In this case the flakes were defatted and disintegrated without removal of the hull fraction. The product had a water content of 7.9% and contained (as calculated on an oven dry basis) 6.9% nitrogen and 1.1% phosphorus, 70% of which was analyzed as phytic acid.

Rapeseed meal of other types. Three samples (each of about 400 g) of rapeseeds, conditioned at room temperature to a water content of 8%, were subjected to different heat treatments. Two of the samples were heated at 90 ^{o} in an externally heated rotating drum, one for 5 minutes, the other for 20 minutes. The third sample was dipped in boiling water for 4 minutes. The heated seeed samples were then converted to a defatted, dehulled rapeseed meal as described for the meal of type A above. The nitrogen and phosphorus contents of the meals were almost identical with those of type A.

Extraction

A weighed amount of rapeseed meal was dispersed in an amount of deionized water, calculated to give a final meal to liquid ratio of 1/20. The pH of the slurry was adjusted to the predetermined value by the addition of aqueous sodium hydroxide or hydrochloric acid. In a few experiments aqueous $CaCl_2$ was added before the pH adjustment. With small samples (2.5 g of rapeseed meal) the final extraction pH was reached within 2.5 minutes and with larger samplex (50 g) within 5 minutes after addition of the meal to water. Insoluble material was removed by centrifuging twice, each time for 5 minutes at 1 400 x g (Wifug X1 with swing-out rotor). The supernatant was analyzed for nitrogen, phosphorus and phytic acid. Nitrogen was determined by the Kjeldahl technique, phosphorus according to Chen et al. (1956) and phytic phosphorus using the method of Wheeler and Ferrel (1971). The amounts of these substances dissolved were calculated as:(concentration of the substance in the supernatant) x (total volume of liquid added).

Precipitation

Hydrochloric acid was added, with thorough stirring to the meal extract until the pre-determined pH of the mixture was reached. In some experiments sodium phytate (BDH biochemicals) was added before the addition of hydrochloric acid. The precipitate was recovered by centrifugation for 5 minutes at 3 100 x g (Wifug X1 with swing-out rotor). The sediment was weighed and its dry matter content (DMC) determined after drying at 85 $^{\circ}$ overnight.

The nitrogen content of the isolate was determined by direct analysis (Kjeldahl) on the dried precipitate. Protein contents were calculated as 5.5 times the nitrogen content (Tkachuk, 1969). The nitrogen yield was calculated as: (total amount of nitrogen in the precipitate)/(total amount of nitrogen in the quantity of extract used to prepare the precipitate). Phosphorus contents in the precipitates were determined as: (total amount of phosphorus in the extract used to prepare the precipitate) - (concentration of phosphorus in the supernatant) x (total volume of liquid added).

RESULTS AND DISCUSSION

Dissolution of substances containing nitrogen and phosphorus

The influence of pH on the relative solubility of the nitrogen containing substances in rapeseed meal of types A and B is shown in Figure 1. The nitrogen solubility curves have a rather complex shape. This can be ascribed, at least partly, to the fact that rapeseed has a very complicated protein composition and contains proteins with iso-electric points in the pH-range 4 to 11 (Lönnerdal, 1975). It should be realized that the curves reproduced in Figure 1, do not represent equilibrium data. The detailed shape of the curves, therefore, might in part be determined by the morphology of the raw material and the time scale of the dissolution experiment. The two rapeseed meals showed relatively high nitrogen solubilities even at their respective solubility minima at pH about 4. From these results it is evident that rapeseed behaves quite differently from more well-known proteinrich oil seeds like soybeans, peanuts and sunflower (cf. e.g. Fontaine et al., 1946).

According to Figure 1, the type B rapeseed meal had a lower nitrogen solubility than that of type A. The difference, which, as can be seen was largest at intermediate pH values, can almost exclusively be ascribed to the effect of the heat treatment step applied in the preparation of the type B meal. The effect of heat treatment of the seeds will be discussed in more detail in the subsequent section.

In preliminary experiments on the nitrogen solubility of rapeseed, it was observed that more or less pronounced, white bands appeared in the sediments obtained by centrifugation of the aqueous meal dispersions. Analysis of such bands showed that they contained high amounts of mineral salts (mainly Mg, Ca, K, and Na) of phytic acid. These findings made it interesting to study also the dissolution of phosphorus containing substances and of phytic acid from the rapeseed meals. Results from such experiments have been included in Figure 1. As can be seen, the curves for the solubility of total phosphorus and of phytic acid run almost parallel. This is understandable, because phytic acid is the dominant phosphorus containing substance in rapeseed. The influence of pH upon the solubility of the phytic acid is rather complex. It can be explained, however, by considering the fact that phytic acid can react with metal ions as well as with proteins. Several authors (Fontaine et al., 1946; Hill and Tyler, 1954; Smith and Rackis, 1957) have reported that phytic acid at low pH values can react with proteins, yielding insoluble complexes. The minimum in phytic phosphorus solubility around pH 2 is probably due to the formation of insoluble protein complexes.

It is known that Ca and Mg salts of phytic acid are soluble at pH values lower than about 4.5 and 6.5 respectively and practically insoluble at higher pH values (Jackman and Black, 1951). The two rapeseed meals tested contained sufficient amounts of Mg and Ca to permit the complete conversion of its phytic acid content into insoluble salts. Hence, the decrease in phytic acid solubility between pH 4.5 and 8 can be explained by the formation of Mg and Ca salts of phytic acid. This is also in agreement with the observation of the appearance of white bands in the sediments as referred to above. With reference to the known solubility behaviour of Ca and Mg phytates, the increase in phytic acid solubility between pH 8 and 10 indicates the formation of strong complexes with the proteins responsible for the concomitant increase in nitrogen solubility. According to this explanation, the decrease in phytic acid solubility in going from pH 10 to 11 would indicate these complexes to be instable at pH values higher than about 10. This is understandable on the following grounds. Rapeseed contains approximately equal amount of arginine and lysine, which are the only amino acid residues to be positively charged at pH 10. The pK_a-values of the lysine and arginine residues are 10.53 and 12.48 respectively. This implies that by going from pH 10 to 11, the number of positive charges of the protein molecules will decrease substantially, which should clearly decrease the stability of protein-phytic acid complexes.

In many of the experiments the aqueous meal dispersion upon centrifugation yielded slightly turbid extract solutions. This was true especially in experiments carried out at pH values lower than 3.5 or between 5 and 11. Several authors (Fontaine et al., 1946; Saio et al., 1967) have suggested the turbidity of soybean

protein extracts to be connected with the presence of phytic acid and this was suspected to be the case here also. Figure 2 shows results from an experimental series in which the centrifugation was carried out at different speeds. As can be seen, the phytic acid content of the extract decreased with an increase in centrifugal force. As expected, a similar influence of the centrifugation speed on the nitrogen content of the extract was not observed. The results confirm that the turbidity of the extracts was caused by the presence of finely divided insoluble phytic acid derivatives. They also imply that the data for the solubility of phosphorus, reported in Figure 1, might include a contribution from colloidal particles, and, hence, be slightly high.

The curves in Figure 3 show the relative solubility of the non-phytic phosphorus in the two meals. The curves were calculated from the smoothed curves given in Figure 1. The experimental points included in the diagram originate from control experiments in which total phosphorus and phytic acid were determined on the same samples. These latter experiments were carried out in order to check the presence of some of the maxima which appeared in the calculated curves. The good agreement between the two sets of data suggests that the very complex solubility pattern, given in Figure 3, is real. It is interesting to note that although large differences do exist between the curves in Figure 3, the curves show maxima and minima at about the same pH. Non-phytic phosphorus-containing substances in rapeseed meal have not been studied. Such substances, which could conceivably be present in rapeseed meal, are inorganic, lipid, carbohydrate and nucleic

acid compounds. Although the nucleic acid content of rapeseed meal is unknown, it can be expected to constitute the major proportion of the non-phytic phosphorus content. For wheat flour it has been shown (Bourdet et al., 1967) that nucleic acid phosphorus constitutes the major proportion of the total non-phytic containing phosphorus content. The same can be shown to be true for soybean meal using figures given by Di Carlo et al. (1955) and Saio et al. (1967). It seems likely, therefore, that the complex shape of the curves in Figure 3 is, to a large extent, a result of complex formation between nucleic acids and proteins with different iso-electric points. Complexes between DNA and proteins have been shown (Hofstee, 1964) to be strongly pH dependent, being weakest at high salt concentrations and at pH:s remote from the iso-electric points of the proteins.

The influence of Ca or Mg salts on the solubility of nitrogen and phytic acid was studied in experiments at pH 8.1. Phytic acid forms insoluble salts with calcium or magnesium at pH 8 (Jackman and Black, 1951). The results in Figure 4 were obtained in experiments with calcium chloride. As expected, addition of calcium chloride resulted in a reduction of the dissolution of phytic acid. As can be seen, the nitrogen solubility increased with an increase in the concentration of calcium. Separate experiments showed that almost identical results were obtained by addition of magnesium chloride instead of calcium chloride.

Influence of a heat treatment

Results which illustrate the effect of a heat treatment of the seeds on some of the properties of the defatted material are given in Table 1. All data in this table are from experiments with dehulled seeds. The mildest heat treatment tested, 5 minutes at 90 °, according to Appelquist and Josefsson (1967) should be sufficient to give an almost total myrosinase inactivation. The intermediate treatment corresponded closely to the treatment used in preparing rapeseed meal of type B, which was used in the experiments underlying Figure 1 b.

As was also seen from the results in Figure 1, the relative decrease in nitrogen solubility produced by a heat treatment was much smaller at pH 11 than at the other pH-values tested. The results show that it is possible to retain a high nitrogen solubility after a myrosinase inactivation step. The results also show, however, that the nitrogen solubility may be seriously diminished if the heat treatment is too severe. It is also seen from Table 1 that a heat treatment resulted in a decrease in the solubility of phytic acid at pH 4.8. At the other pH-values, which corresponded to phytic acid solubility minima (cf. Figure 1), the effects were small or not detectable.

It was also observed that a heat treatment of the seeds markedly changed the buffer capacity of the resulting meal. Results illustrating this effect have been included in Table 1, which shows the amount of sodium hydroxide, required by aqueous meal slurries containing 1 g of meal to reach pH 11.1 or 8.2, or the

amount of hydrochloric acid, required to reach pH 4.8. The mechanism responsible for the observed effect has not been fully elucidated. It is most likely, however, that the effect is a consequence of heat induced processes in the proteins, similar to those observed in meat, where it has been shown that a heat treatment results in an uncovering of histidine groups, making the protein more basic (Conell and Howgate, 1964; Hamm, 1965).

Figure 5 shows that the decrease in the nitrogen solubility produced by a heat treatment of the seeds, to a certain extent could be overcome by working at a higher extraction temperature. It should be noted, however, that in the experiments underlying Figure 5, pH was measured at the extraction temperature. This means that the amount of sodium hydroxide present in the extracts was higher at the higher extraction temperatures. This difference in electrolyte concentration will affect the possibility of recovering the dissolved proteins by precipitation.

Protein extraction at pH 6.2. Figure 6 shows the influence of the pH of precipitation on the recovery of dissolved nitrogen and phosphorus, on the dry matter content (DMC) of the precipitates (as determined on sediments collected by a standard centrifugation technique) and on the protein content of the precipitates. It can be seen that about 50 per cent of the extracted nitrogen could be recovered by acid precipitation.

A characteristic feature was that the dry matter content of the precipitates was strongly dependent on the pH of precipitation, and went through a maximum, which occurred at a pH considerably higher than that at which the nitrogen recovery reached its maximum value. Although a clear understanding of this phenomenon has not yet been arrived at, results to be discussed in the next section strongly support the assumption that the maximum in DMC was caused by the presence of phytic acid in the extract. It should be pointed out, however, that the maximum in DMC was not paralleled by a maximum in the phosphorus content of the precipitates. The amount of phosphorus in the precipitates continuously increased with a decrease in the pH of precipitation. Thus, the P/N-ratio (w/w) of the precipitates increased from 0.06 at pH 4.8, where the maximum in DMC occurred, to 0.15 at pH 3. At the latter pH about 50 per cent of the phosphorus in the extract was bound to the precipitate (Figure 6).

Protein extraction at pH 8.2. As can be seen from Figure 1, extraction at pH 8.2 gave an extract solution containing rather small amounts of phosphorus. This made it possible to study the effect of phytic acid on the precipitation behaviour of the extracted proteins. Figure 7 shows results from experiments with the extract as obtained (Figure 7 a) and with an extract to which sodium phytate was added (Figure 7 b). The amount of sodium phytate added was calculated to give a phytic acid concentration equal to that of the extract solution prepared at pH 6.2.

As can be seen, addition of sodium phytate resulted in a most pronounced broadening of the maximum in the nitrogen precipitation curve and in a slight increase in the maximum recovery of nitrogen. The maximum recovery as well as the protein content of the precipitates (not shown in the diagram) were almost the same as those given in Figure 6.

Addition of sodium phytate had a large effect on the DMC of the precipitates. Also in this case, the maximum in DMC occurred at pH 4.8, a pH value which was just outside the pH range of maximum nitrogen recovery. The agreement between the results obtained in the experiments with added phytate and those given in Figure 6 is striking.

Figure 7 c gives results from experiments with a solution prepared by extraction at pH 8.2 in the presence of 0.04 M $CaCl_2$. In this case, the precipitation maximum was very narrow and only about 25 per cent of the dissolved proteins could be recovered. These results show that the positive effect of calcium on the protein dissolution step (Figure 4) was paralleled by a negative effect on the precipitation step. The influence of calcium chloride on the precipitation step can partly be ascribed to the general effect of electrolytes on electrostatic interactions within and between the protein molecules, and partly to its effect on the composition of the extract solution, notably the phytic acid content.

Extraction at pH 11.1. With reference to the solubility behaviour of the proteins (cf. Figure 1) a technical process for the preparation of a rapeseed protein isolate most likely has to be based on a protein extraction step conducted at a fairly high pH. This is especially true if the seeds to be processed have been subjected to a heat treatment. Results from precipitation experiments with extract solutions prepared at pH 11.1 are given in Figure 8. The pH used was chosen to give an extract solution containing a minimum of phosphorus containing substances. As the results show, the precipitation maximum in this case was very broad, covering a much wider pH range than the precipitation maximum in Figure 7 a. This, most probably, is partly a consequence of the fact that the extract contained a large number of different proteins having widely different molecular weights and iso-electric points. It is likely, however, that interactions between proteins and other seed components in the extract (e.g. carbohydrates and nucleic acids) also played a role in determining the observed precipitation behaviour.

The maximum yield was rather low, and more than 50 per cent of the dissolved proteins remained in the supernatant. Attempts to study the feasability of increasing the yield by carrying out the precipitation in two successive steps were made. In the first step, hydrochloric acid was added to lower the pH to 6.6. The supernatant obtained after removal of the precipitate was then used to study the influence of the pH of precipitation on the recovery of nitrogen in the second step. Results from these latter experiments are given in Figure 8 b.

As can be seen, precipitation occurred in a rather limited pH range only. The maximum recovery in the second step amounted to 18 per cent of the dissolved nitrogen. This means that the theoretical maximum yield of nitrogen in this two step precipitation process was 63 per cent. The results obtained are in agreement with the high solubility of rapeseed protein (cf.Figure 1) and with previous literature data. Thus, Girault (1973) has reported a maximum recovery of 56 per cent of the dissolved proteins, Pókorny (1963) 49 per cent and Gillberg and Törnell (1970) 55 per cent.

The results reported in Figure 8 were obtained using a rapeseed meal of type A as a raw material. Experiments with rapeseed meal of the type B gave rather similar results (Figure 9, broken curves). The main difference consisted in a displacement by about 0.5 to 1 pH unit of the nitrogen precipitation curve towards higher pH values. This may reflect an influence of the heat treatment on the apparent basicity of the proteins as discussed above. In experiments with fractionated precipitation it was found that the maximum yield in the second precipitation step corresponded to 7 per cent of the extracted nitrogen. The precipitation maximum in the second step occurred at pH 4.3, which was about 0.5 pH units higher thant that of the maximum in Figure 8 b. The theoretical total nitrogen yield in the two steps was slightly lower than 50 per cent.

As was found in the experiments with extract solutions prepared at pH 8.2, addition of sodium phytate strongly affected the precipitation behaviour of the extract. Thus, it was found that the nitrogen recovery obtained by precipitation to pH 4.9 could be increased from 34 to a maximum of 78 per cent by the addition of sodium phytate in an amount correspondning to 0.055 g of phytic phosphorus per g of nitrogen. The results in Figure 9 are from experiments with an extract to which had been added 0.049 g of phosphorus in the form of sodium phytate per g of dissolved nitrogen, that is, an addition of sodium phytate, slightly lower than that which gave a maximum in nitrogen recovery at pH 4.9. As can be seen, addition of sodium phytate in this case brought about a narrowing of the precipitation maximum. It is interesting to note that the increasing of the yield had no negative effect on the nitrogen content of the precipitates. The P/N-ratio in the precipitates increased as the pH of precipitation was lowered. Thus, at pH 5.0, the P/N-ratio was 0.084 and at pH 3 it was 0.128 (w/w). These results imply that the phosphorus containing substances which entered into solution during the extraction step were incorporated in the precipitate to about the same extent as was the phytate added. The presence of sodium phytate had a favourable effect also on the DMC of the product. In this case, the maxima in DMC and in nitrogen recovery occurred at about equal pH values.

The results presented show that the direct precipitation by acid of alkaline rapeseed extracts give protein isolates in rather low yields. The nitrogen recovery could be considerably improved, however, by conducting the precipitation step in the presence of sodium phytate. This also brought about an increase in the dry matter content of the precipitate, an effect which is technically favourable as it simplifies the separation and the washing of the product. It is known, however, that phytic acid has an adverse effect on the utilization of calcium, iron, zinc and other polyvalent metal ions in the food (Reinhold et al., 1973) and that it can also exert an inhibiting effect on the enzymatic degradation of proteins (Barré, 1956). From a nutritional point of view, therefore, sodium phytate would not be an attractive aid for the precipitation of proteins. For this reason, attempts to find other substances which can be used as precipitation aids have been made. Some of the work along this line will be presented in the next paper in this series.

REFERENCES

Appelqvist, L.-Å., Josefsson, E. 1967. Method for quantitative determination of isothiocyanates and oxazolidinethiones in digests of seed meals of rape and turnip rape. J. Sci. Food Agr. 18(11):510.

Barré, R.M. 1956. Influence de l´acide phytique sur la digestion pepsique de différentes proteines. Ann. pharm. franc. 14:182.

Bourdet, A., Feillet, P. 1967. Distribution of phosphorus compounds in the protein fractions of various types of wheat flours. Cereal Chem. 44(5):457.

Di Carlo, J., Schultz, A.S., Kent, A.M. 1955. Soybean nucleic acid. Arch. Biochem. Biophys. 55:253.

Chen, P.S., Toribara, T.Y., Warner, H. 1956. Microdetermination of phosphorus. Anal. Chem. 28(11):1756.

Conell, J.J., Howgate, P.F. 1964. The hydrogen ion titration curves of native, heat-coagulated and frozen-stored myofibrils of cod and beef. J. Food Sci. 29:717.

Fontaine, T.D., Pons. W.A., Jr., Irving, G.W., Jr. 1946. Protein-phytic acid relationship in peanuts and cottonseed. J. Biol. Chem. 164:487.

Gillberg, L., Törnell, B. 1970. "Framställning av spinnbart rapsproteinisolat". Discourses at the proteinday, p. 70. Dept. of Food Technology, Chemical Center, Lund, Sweden.

Girault, A. 1973. The study of some properties of rapeseed protein with a view to protein concentrate production. J. Sci. Fd. Agric. 24:509.

Hamm, R. 1965. Heating of muscle systems. In Ed. Brieskey, E.J., Cassens, R.C., Trautman, J.G. The physiology and biochemistry of muscle as a food, p. 363. University of Wisconsin Press, Madison, USA.

Hill, R., Tyler, C. 1954. The reaction between phytate and protein. J. Agr. Sci. 44:324.

Hofstee, B.H.J. 1964. Solubility and composition of protein-deoxyribonucleic acid complexes. Biochim. Biophys. Acta 91:340.

Jackman, R.H., Black, C.A. 1951. Solubility of iron, aluminum, calcium and magnesium inositol phosphates at different pH values. Soil Sci. 72:179.

Josefsson, E., Appelqvist, L.-Å. 1968. Glucosinolates in seed of rape and turnip rape as affected by variety and environment. J. Sci. Food Agric. 19(10):564.

Kodagoda, L.P., Young, C.Y., Nakai, S., Powrie, W.D. 1973. Preparation of protein isolates from rapeseed flour. Can. Inst. Food Technol. J. 6(3):135.

Lönnerdal, B., Jansson, J.-C. 1972. Studies on Brassica seed proteins. I. The low molecular weight proteins in rapeseed. Isolation and characterization. Biochim. Biophys. Acta 278:175.

Lönnerdal, B. 1975. Dept. of Nutr., University of Uppsala, Sweden. To be published.

Owen, D.F., Chichester, C.O., Granadino, C., Mönckeberg, F. 1971. A process for producing nontoxic rapeseed protein isolate and an acceptable feed by-product. Cereal Chem. 48(2):91.

Pokorný, J., Vodička, M., Žalud, J. 1963. Rapeseed III. The precipitation of rapeseed proteins from alkaline solutions with diluted acids. Sci. Papers Inst. Techn. Prague, Food Techn. 7(2):167.

Reinhold, J.G., Nasr, K., Lahimgarzadeh, A., Heydayati, H. 1973. Effects of purified phytate and phytate rich bread upon metabolism of zinc, calcium, phosphorus, and nitrogen in man. The Lancet 10:283.

Saio, K, Koyama, E., Watanabe, T. 1967. Protein-calcium-phytic acid relationship in soybean. Part I. Effects of calcium and phosphorus on solubility characteristics of soybean meal protein. Agr. Biol. Chem. 31(10):1195.

Shaikh, I.A., Arshad, M., Haque, M.Y., Ali, S.M. 1968. Nutritional properties of rapeseed protein concentrate prepared from commercial rapeseed cake. Pakistan J. Sci. Ind. Res. 11:263.

Smith, A.K., Rackis, J.J. 1957. Phytin elimination in soybean protein isolation. J. Am. Chem. Soc. 79:633.

Sosulski, F.W., Bakal, A. 1969. Isolated proteins from rapeseed, flax and sunflower meals. Can. Inst. Food Technol. J. 2(1):28.

Tkachuk, R. 1969. Nitrogen-to-protein coversion factors for cereals and oilseed meals. Cereal Chem. 46:419.

Wheeler, E.L., Ferrel, R.E. 1971. A method for phytic acid determination in wheat and wheat fraction. Cereal Chem. 48:312.

Acknowledgements

Thanks are due to Mrs Jana Polackova for valuable experimental assistance, and to Dr Ragnar Ohlson and Dr Klas Anjou for helpful discussions and for providing us with rapeseed. Oil extraction and dehulling of the seeds were carried out at AB Karlshamns Oljefabriker, Karlshamn, Sweden (by courtesy of Dr Anjou). The financial support of The Swedish Board for Technical Development is gratefully acknowledged.

Table 1. Effect of different heat treatments on the buffer capacity and solubility of nitrogen and phytic phosphorus in rapeseed meal.

pH of extraction	Heat treatment of the seeds			
	None	5 min 90 °C	20 min 90 °C	4 min boiling
	NaOH or HCl added to reach the extraction pH (mmol/g meal)			
11.1	0.87	0.58	0.59	0.61
8.2	0.39	0.16	0.16	0.16
4.8 (HCl)	0.12	0.25	0.25	0.26
	Nitrogen solubility (%)			
11.1	94	93	87	43
8.2	62	53	47	19
4.8	57	47	44	22
	Solubility of phytic phosphorus (%)			
11.1	2	2	2	2
8.2	9	5	5	4
4.8	83	72	74	55 ± 10

Text to the figures

Figure 1. Dissolution of nitrogen (O), total phosphorus (x) and phytic phosphorus (Δ), as a function of pH during extraction of rapeseed meal of type A (a) and of type B (b).

Figure 2. Influence of the speed of centrifugation (R.C.F.) on the amount (% of total) of nitrogen (O) and phytic phosphorus (Δ) found in extract solutions obtained by extracting rapeseed meal of type A at pH 8.1.

Figure 3. Dissolution of non-phytic phosphorus from rapeseed meals of types A (——, ⊡) and B (---, Θ) as a function of pH (see text).

Figure 4. Influence of $CaCl_2$ on the dissolution, at pH 8.1, of nitrogen (O) and phytic phosphorus (Δ) from rapeseed meal of type A.

Figure 5. Influence of the temperature of extraction on the dissolution of nitrogen at pH 8.2 (a) and at pH 11.1 (b) from three rapeseed meals, prepared from seeds subjected to different heat treatments.

Rapeseed protein isolates I.

Figure 5.

Symbol	Heat treatment of the seeds
O	None
□	20 min at 90 ^{o}
Δ	4 min in boiling water

Figure 6. Precipitation of a protein extract solution prepared at pH 6.2, using rapeseed meal of type A.

O	Nitrogen yield
x	Phosphorus precipitated (Reported as % of the total amount of phosphorus in the extract)
Δ	DMC of the precipitates
□	Protein content of the precipitates (N x 5.5).

Figure 7. Precipitation of protein extract solutions prepared from rapeseed meal of type A by

a) extracting at pH 8.2

b) extracting as in a) but adding sodium phytate (0.074 g phytic phosphorus per g of nitrogen of the extract) before precipitation.

c) extracting as in a) at pH 8.2 in the presence of 0.04 M $CaCl_2$

O	Nitrogen yield
Δ	DMC of the precipitates

Figure 8. Influence of pH on the precipitation of a protein extract solution, prepared at pH 11.1 from rapeseed meal of type A.

a) precipitation in one step

b) precipitation of the supernatant obtained by first carrying out a precipitation step at pH 6.6.

O Nitrogen yeild (N.B. In diagram b, the percentage of N is expressed as percentage of that in the original extract)

Δ DMC of the precipitates

□ Protein content of the precipitates

Figure 9. Precipitation of a protein extract solution, prepared at pH 11.1 from rapeseed meal of type B, after addition of sodium phytate to the extract (0.049 g phytic phosphorus per gram extracted nitrogen).
(The broken curves represent an experiment in which phytate was not added).

O Nitrogen yield

Δ DMC of the precipitates

□ Protein content of the precipitates

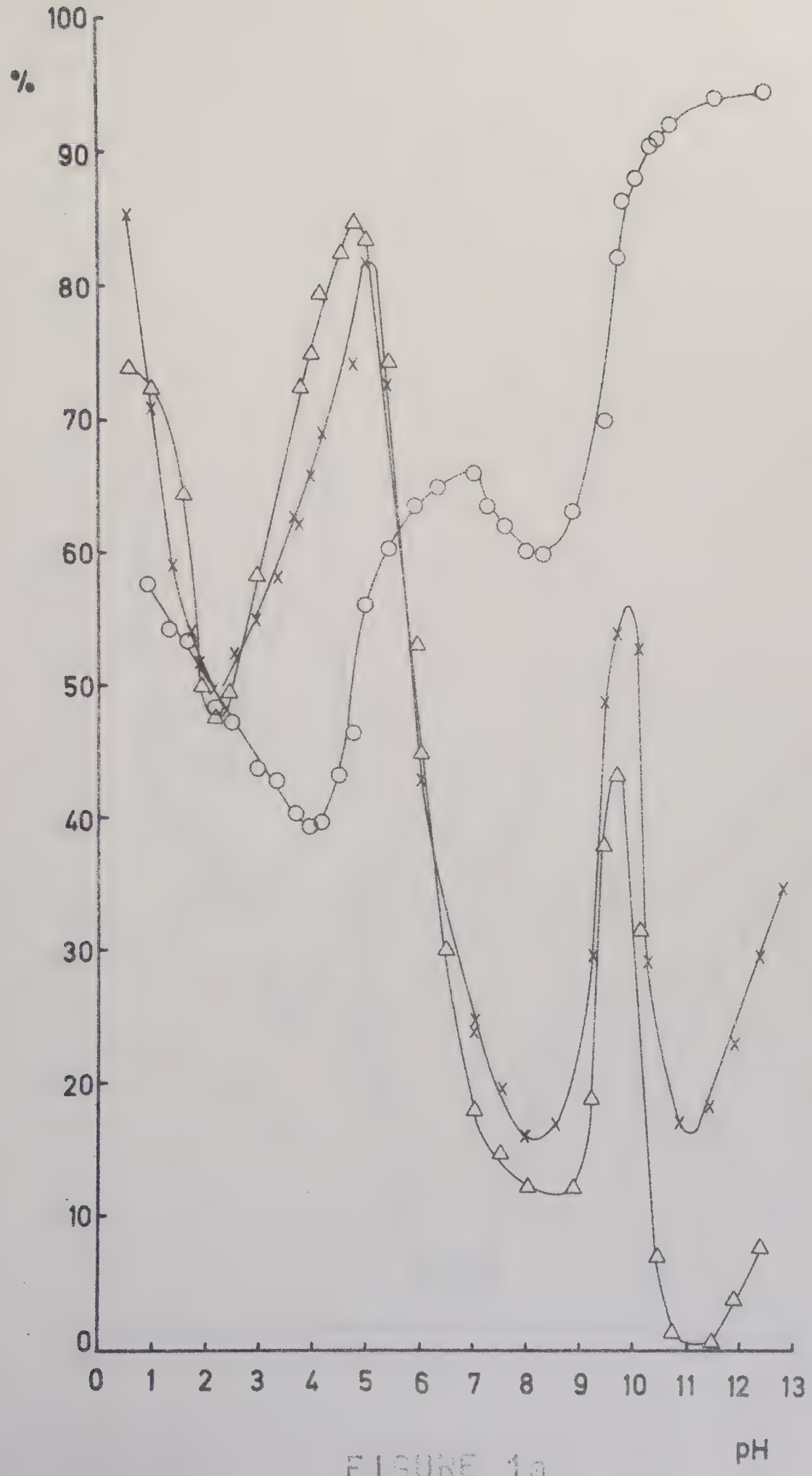


FIGURE 1a

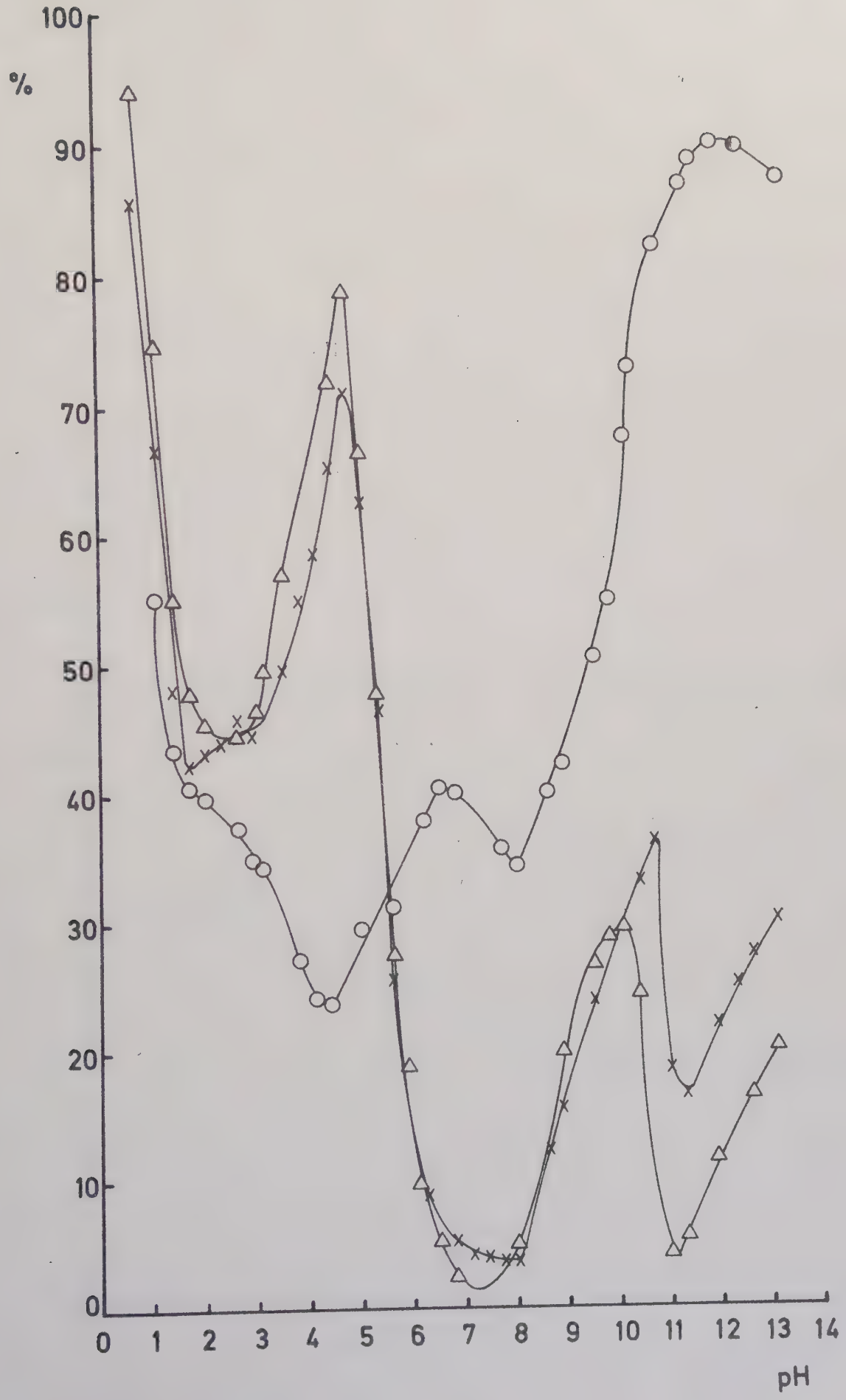


FIGURE 1b

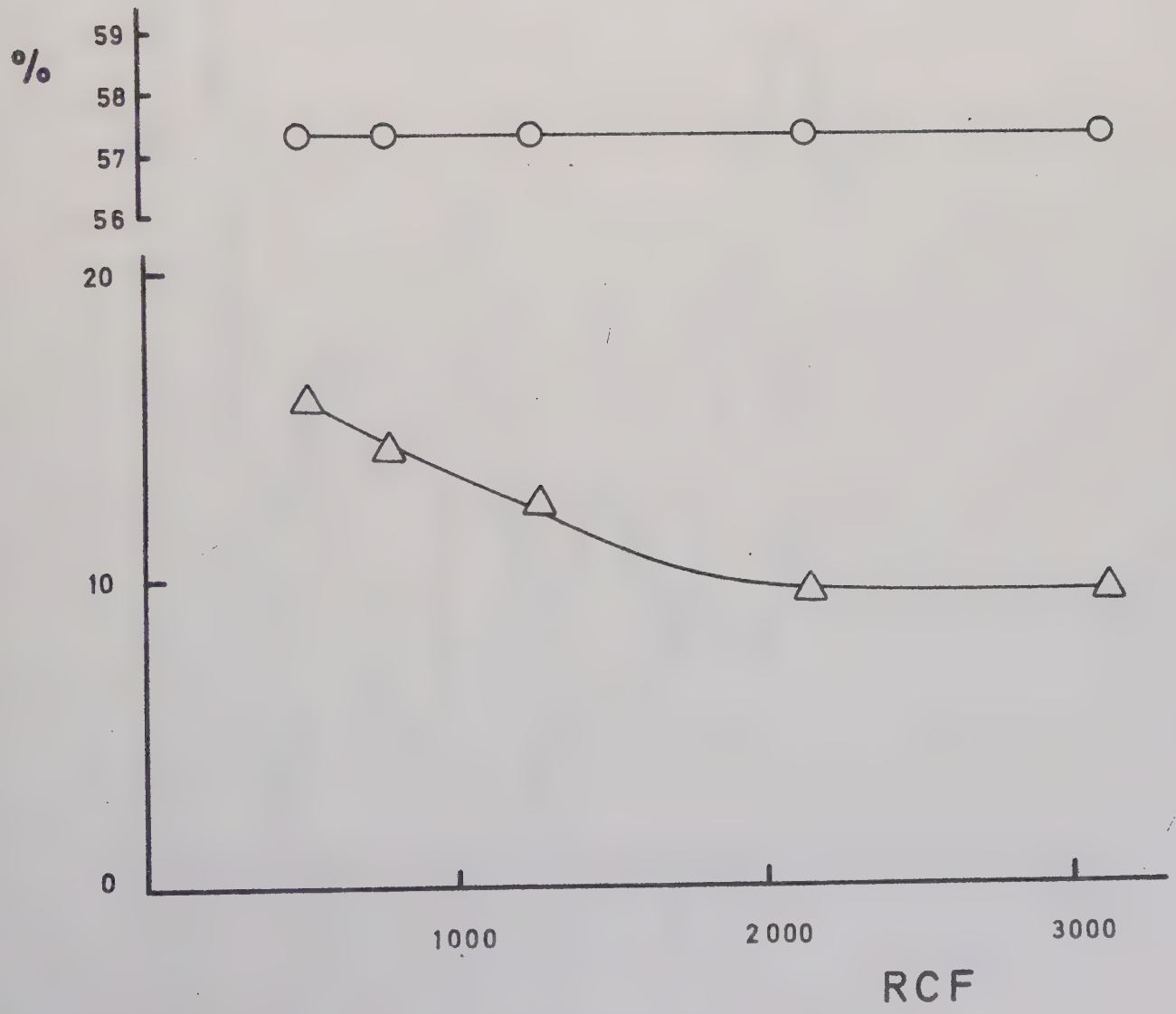


FIGURE 2

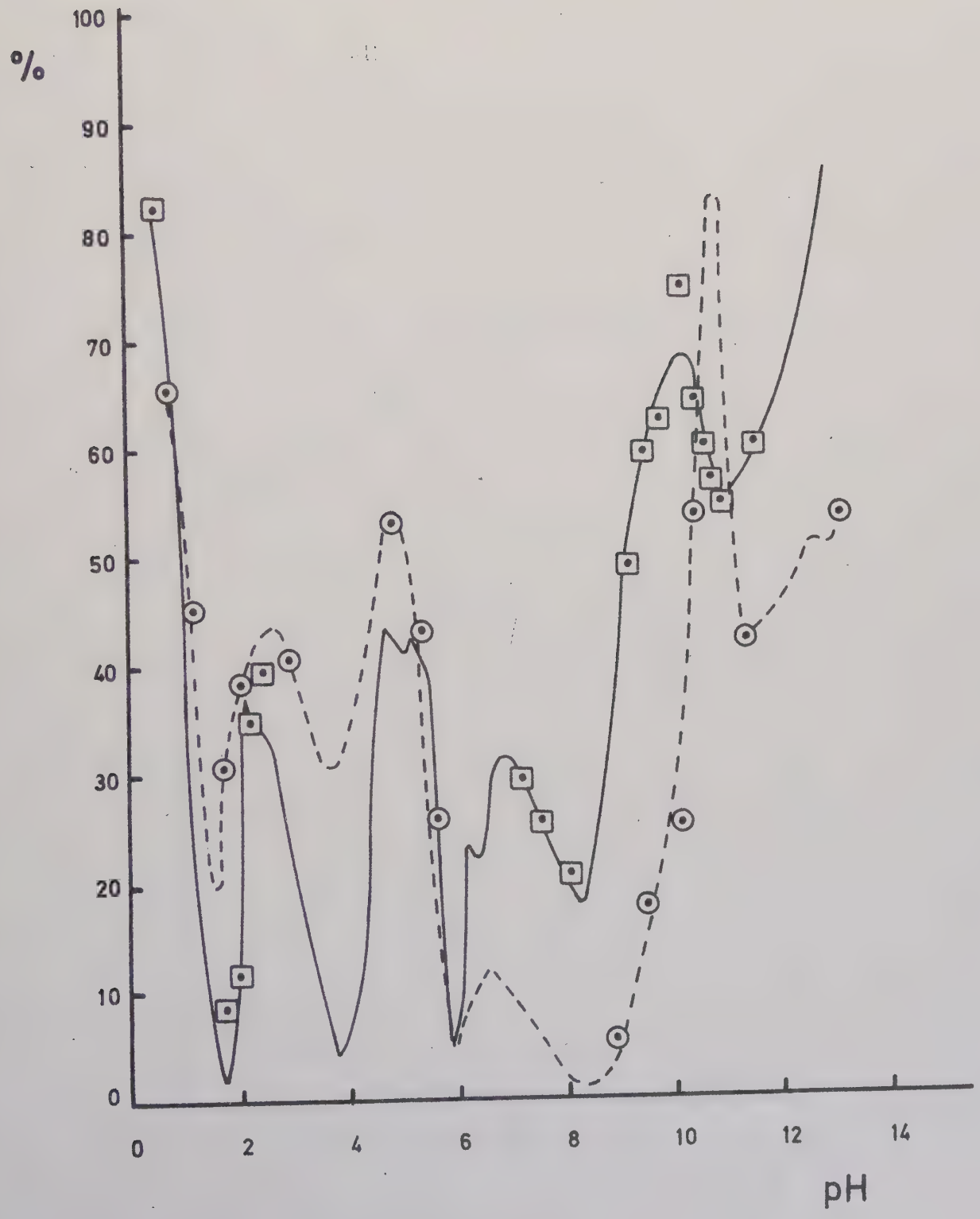


FIGURE 3

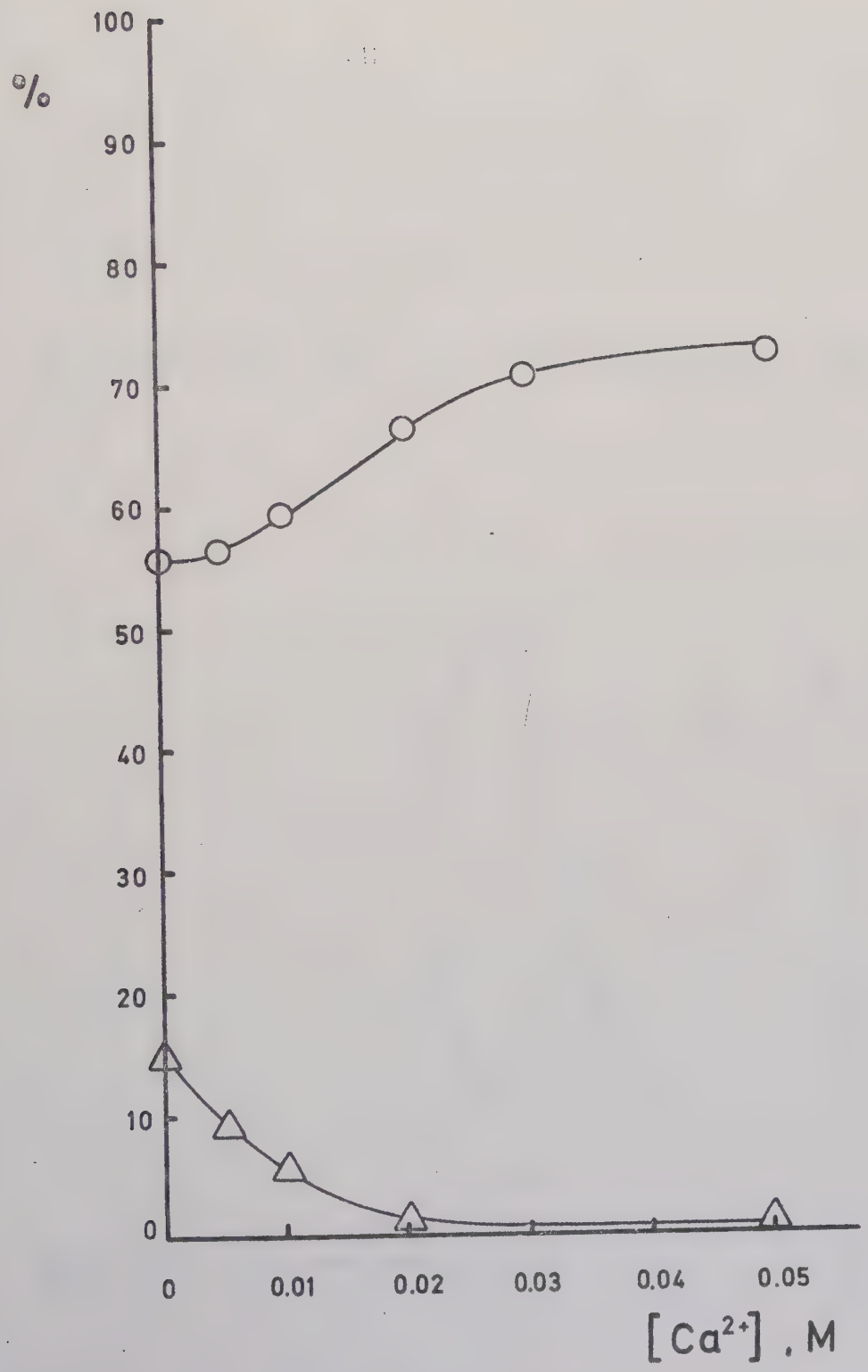


FIGURE 4

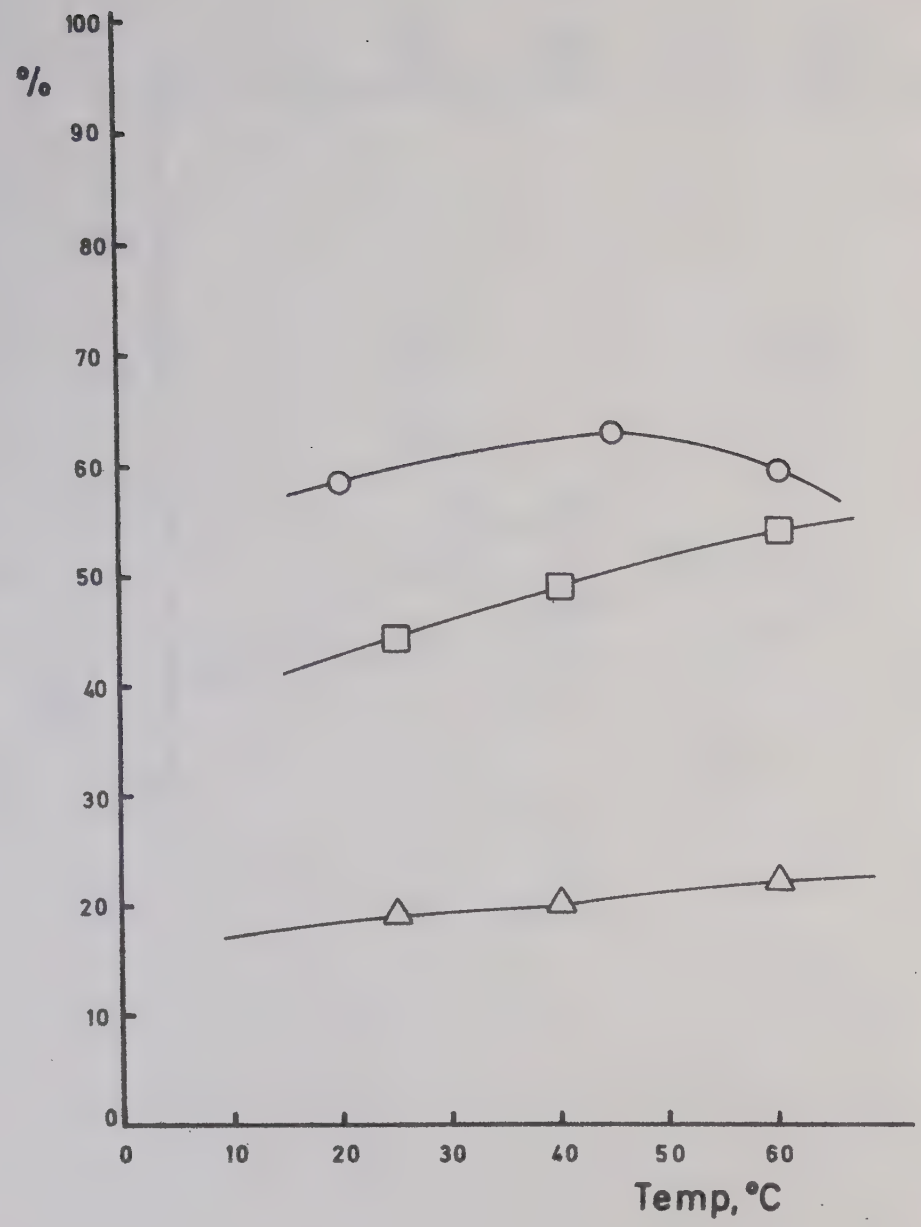


FIGURE 5a

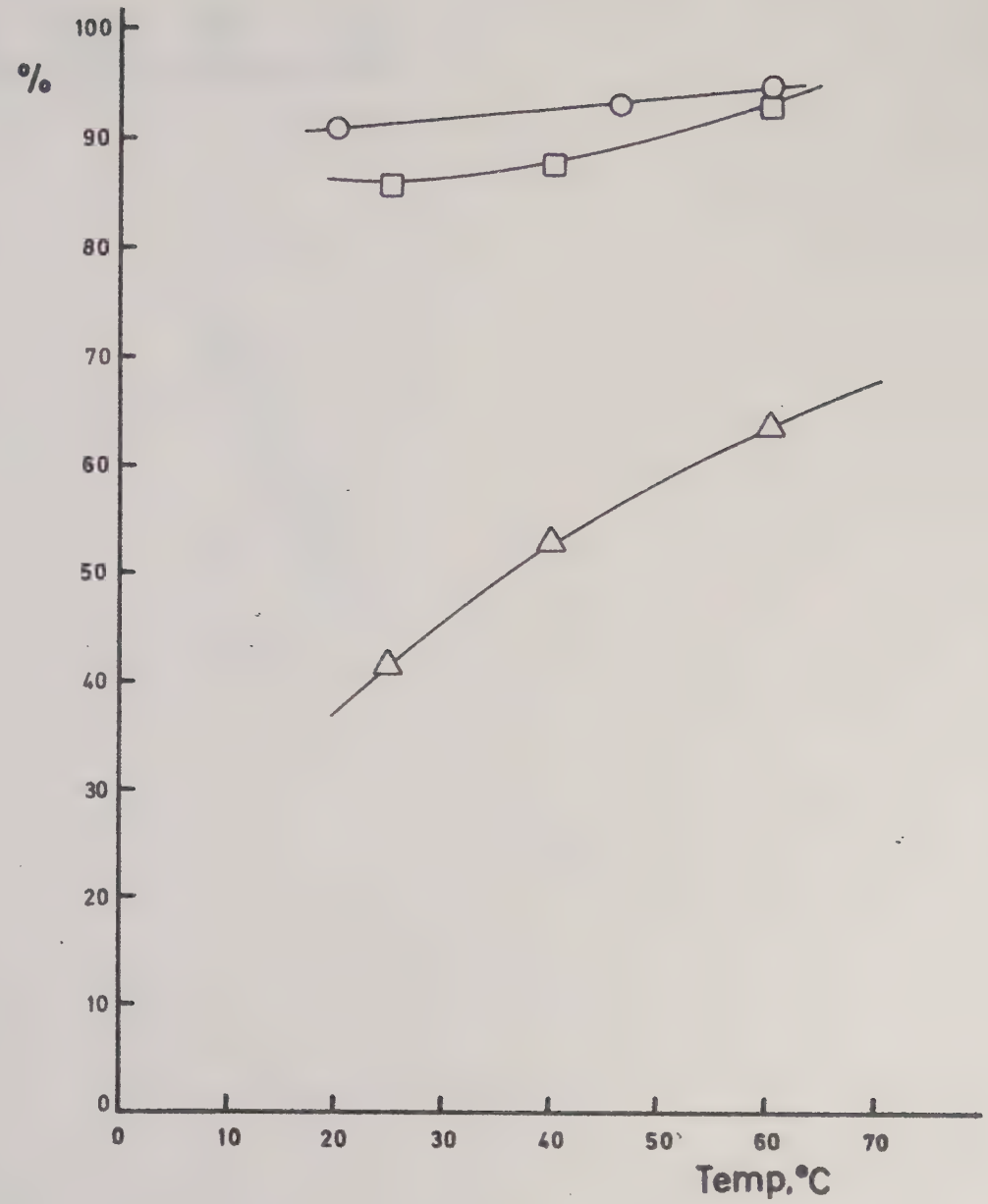


FIGURE 5b

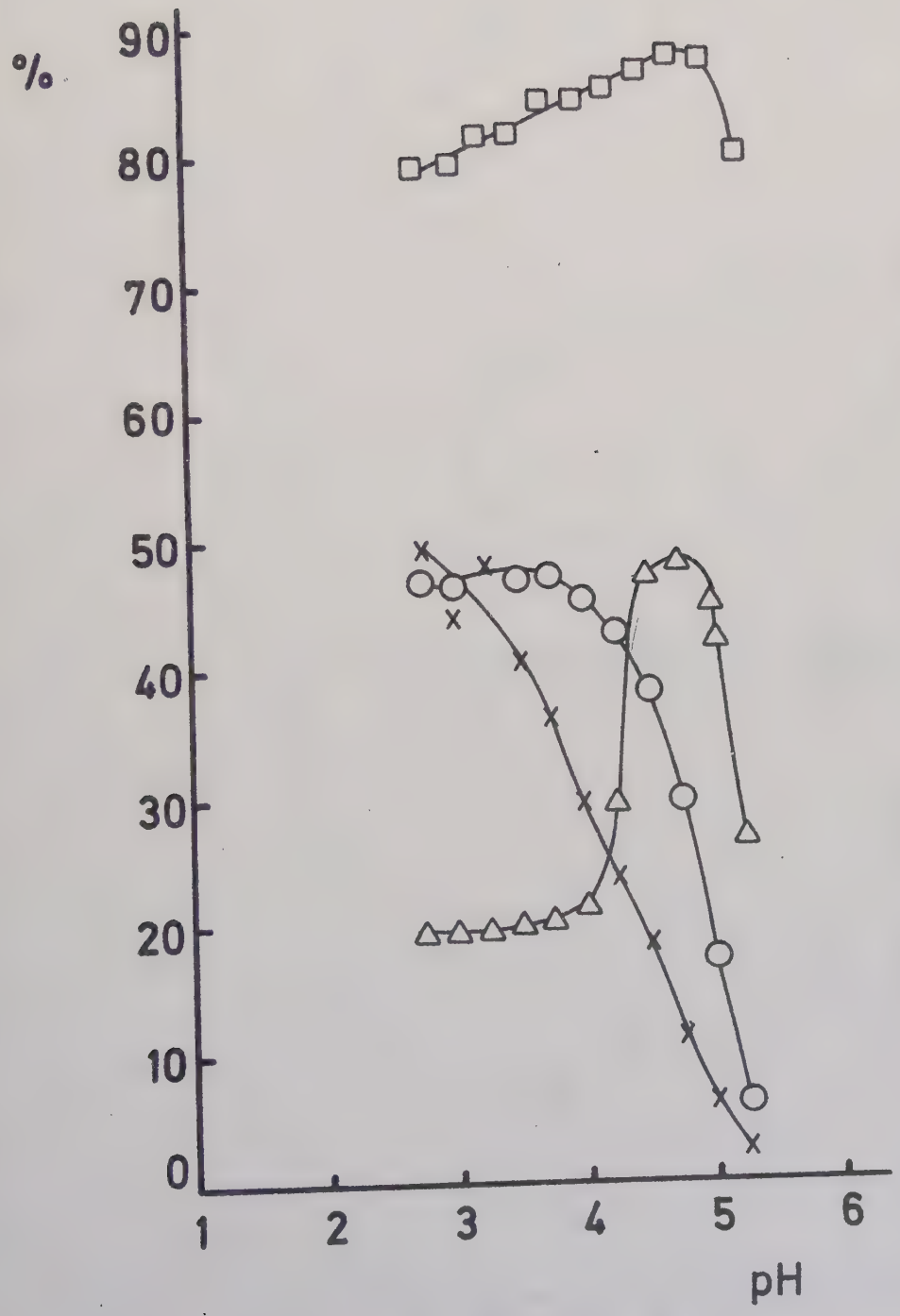


FIGURE 6

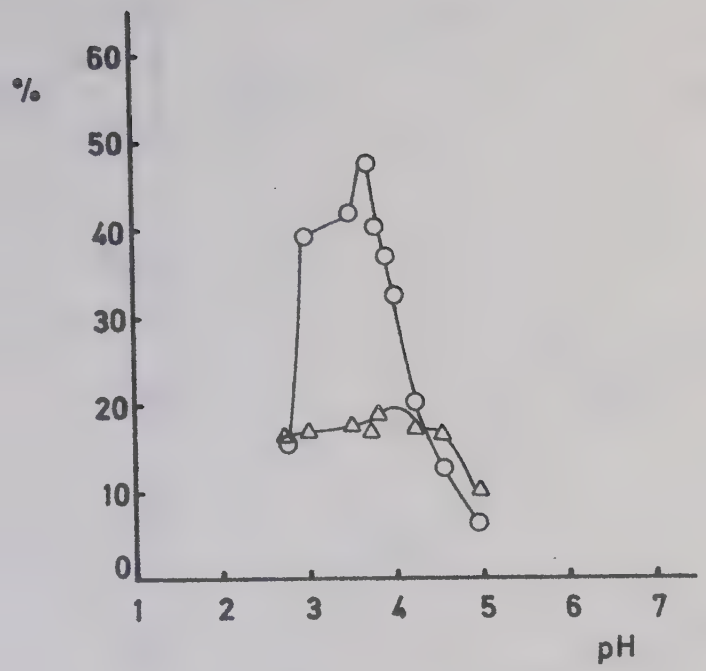


FIGURE 7a

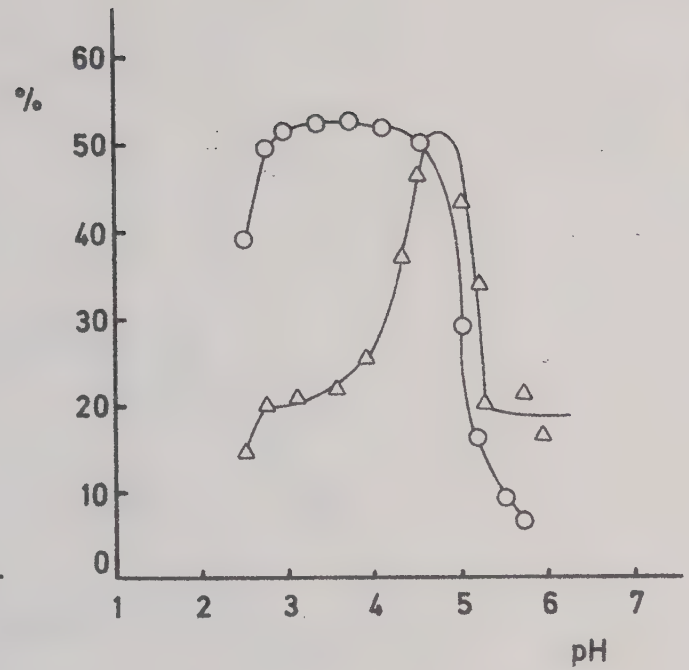


FIGURE 7b

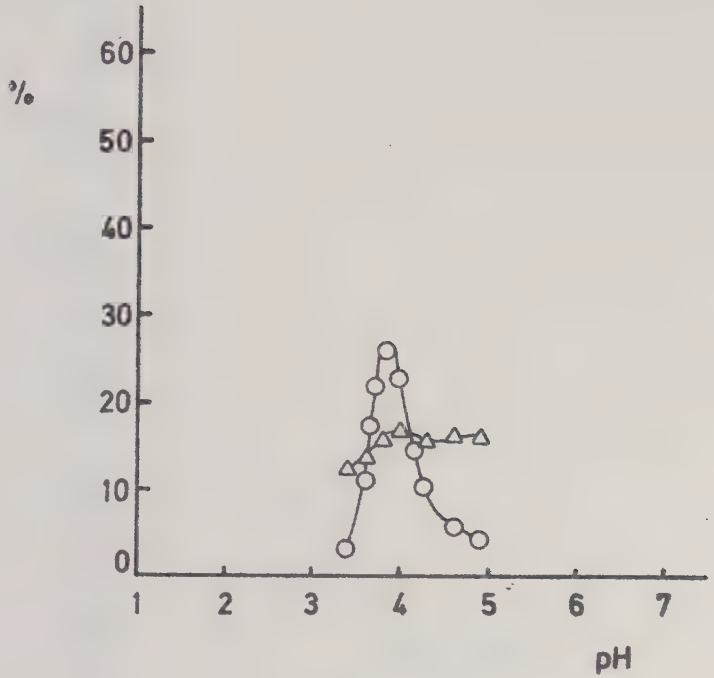


FIGURE 7c

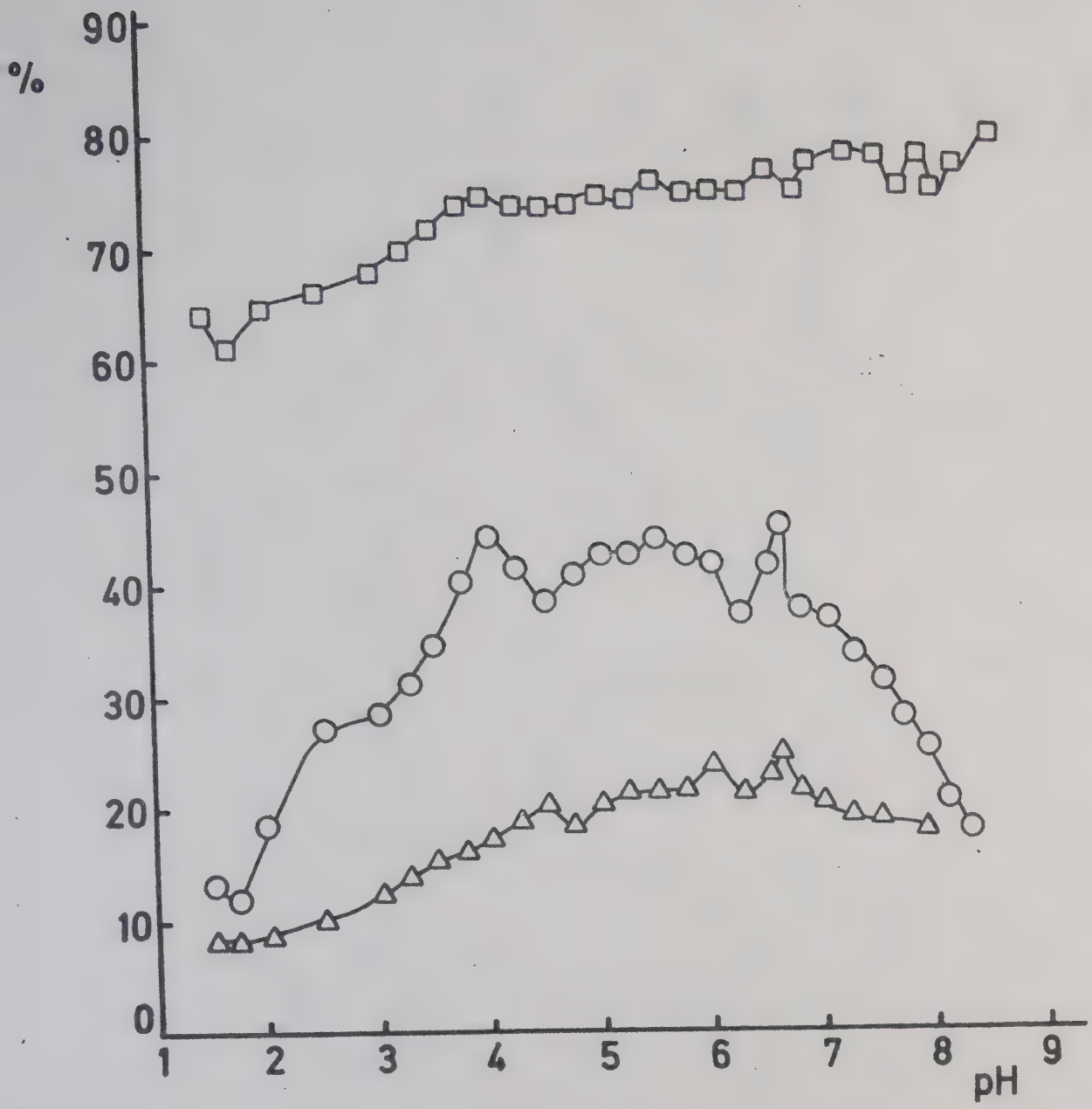


FIGURE 8a

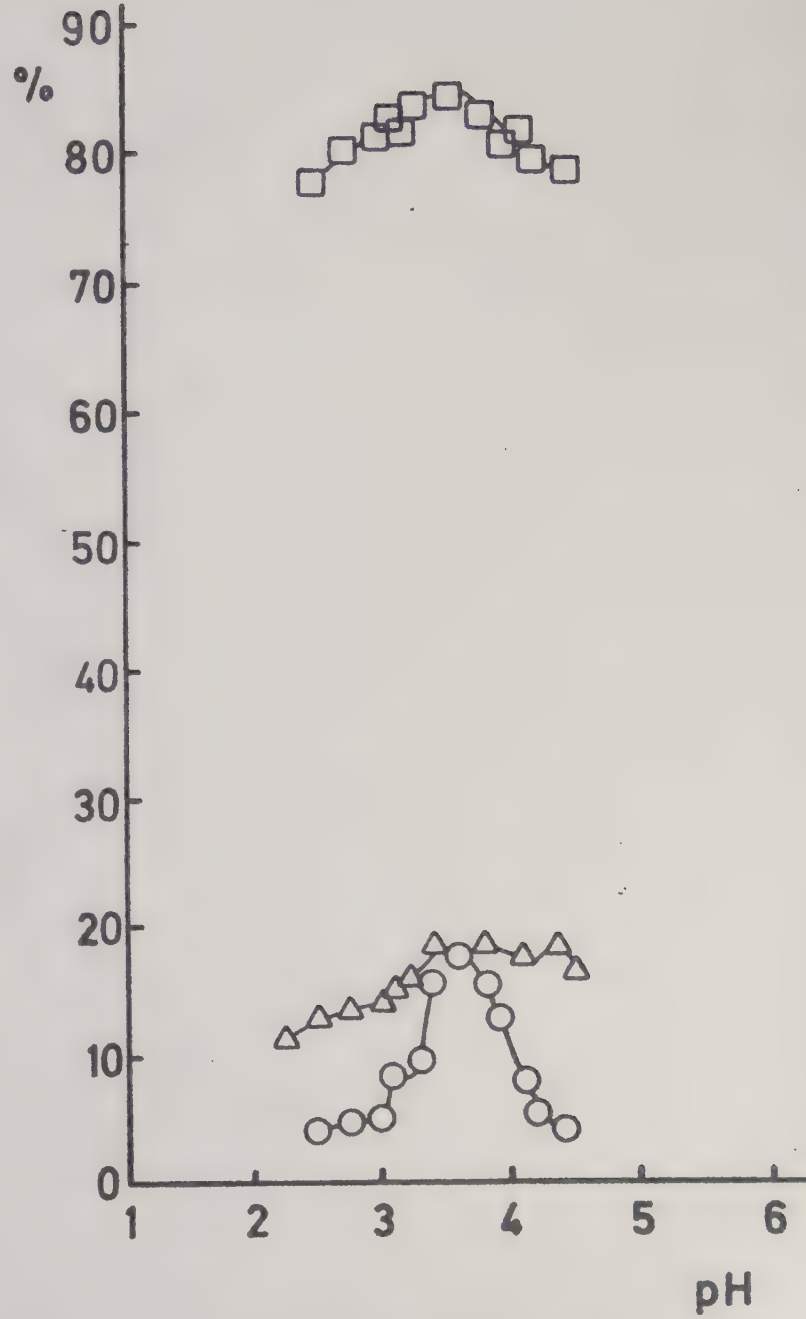


FIGURE 8b

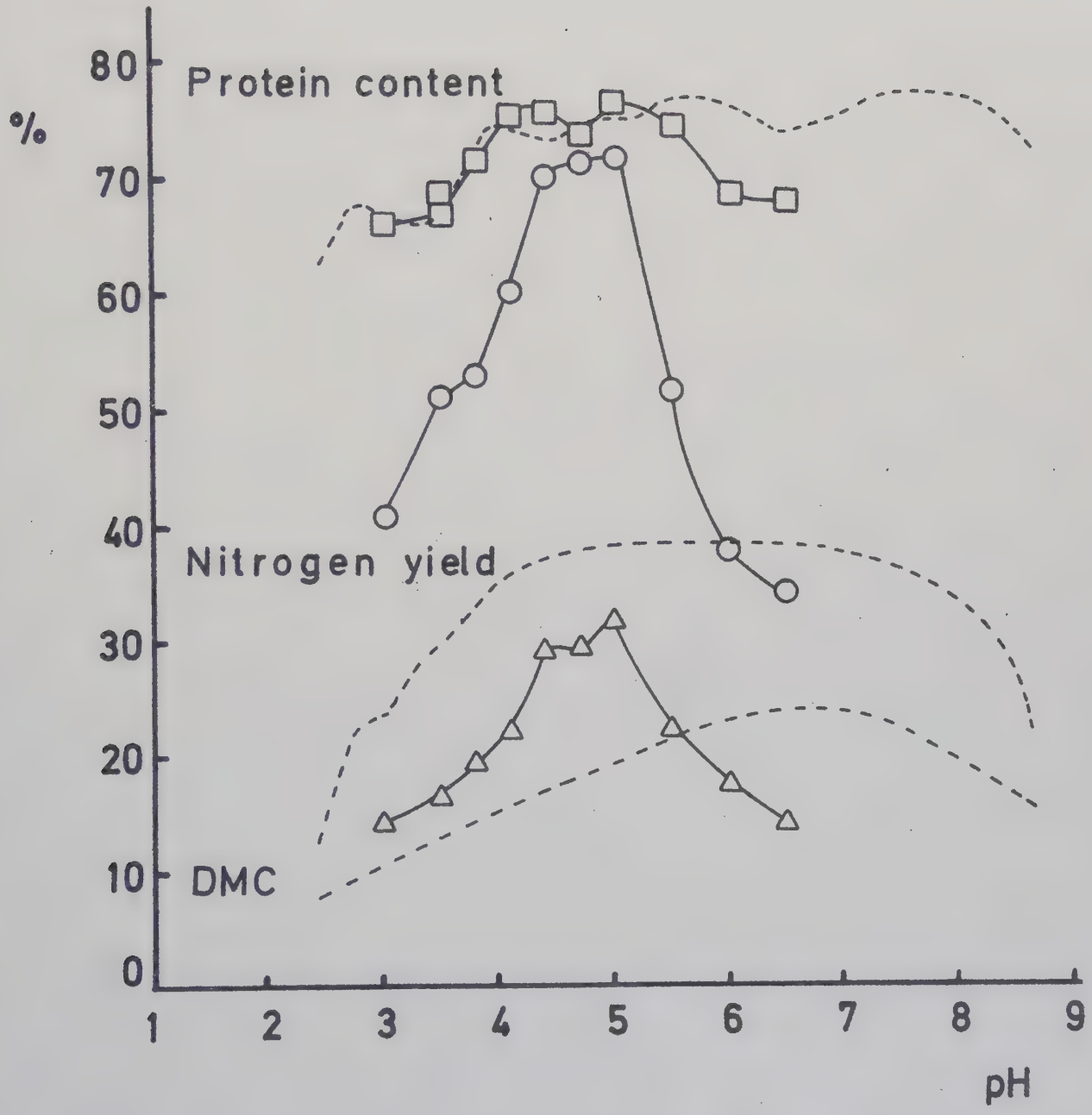

Protein content
Nitrogen yield
DMC
%
80
70
60
50
40
30
20
10
0
1
2
3
4
5
6
7
8
9
pH

FIGURE 9

PREPARATION OF RAPESEED PROTEIN ISOLATES

II. Precipitation of rapeseed proteins in the presence of polyacids.

Lars Gillberg and Bertil Törnell

The Lund Institute of Technology
Department of Chemical Technology
Chemical Center
P.O.B. 740
S - 220 07 Lund 7
Sweden

Rapeseed protein isolates.

PREPARATION OF RAPESEED PROTEIN ISOLATES. II. Precipitation of rapeseed proteins in the presence of polyacids. L. Gillberg and B. Törnell. J. Food Sci.

The precipitation of alkaline extracted rapeseed proteins by addition of acid in the presence of different acidic polymers have been studied. The yield and the nitrogen and dry matter contents of the isolates were found to depend on the ionic composition of the extract, the nature and amount of added polymer and the pH of precipitation. The acidic polymers studied were hexametaphosphate, CMC of different DP and DS, polygalactouronic acid, alginate and kappa-, iota-, and lambda-carrageenans.

With all of these polymers, it was possible to obtain a quantitative or almost quantitative recovery of the dissolved proteins. Except for the carrageenans, all of the polymers produced a high increase in the dry matter content of the prcipitates.

INTRODUCTION

In the first paper in this series, it was shown that the direct precipitation by acid of rapeseed protein extracts, prepared at a pH of about 11, gave protein isolates in rather low yields. It was observed, however, that the nitrogen recovery could be considerably increased by adding sodium phytate to the protein extracts prior to their precipitation. The maximum increase in nitrogen yield was obtained at phytate additions smaller than those corresponding to the amount of phytate present in the quantity of rapeseed meal used to prepare the extract (Gillberg and Törnell, 1975). Precipitation in the presence of sodium phytate also resulted in a very pronounced increase in the dry matter content of the precipitate as recovered by centrifugation. These observations suggest that phytic acid would be useful as a precipitation aid in the preparation of rapeseed protein isolates. Unfortunately, however, phytic acid is likely to exert an adverse effect on the nutritional value of the protein isolate (Barré, 1956; Reinhold et al., 1973; O'Dell and Savage, 1960). It was therefore of interest to study the possibility of obtaining similar favourable effects with other precipitation aids not possessing this drawback.

In the present investigation, the potential use of a number of different polyacids as precipitation aids in the preparation of rapeseed protein isolates have been elucidated.

Phase separation phenomena in systems containing polyanions and proteins have been the subject of many previous studies. Reference should be made to the early and very extensive work by Bungenberg de Jong (1949) on complex coacervation between gelantine and gum arabic and to results presented by Morawetz and Hughes (1952) on interactions between synthetic polyelectrolytes and bovine serum albumin. Among more recent contributions with a special relevance to the present study, can be mentioned that reported by Nitschman et al. (1959) on the use of polyphosphates for the precipitation of plasma proteins and the work by Hidalgo and Hansen (1969) on the precipitation of β-lactoglubulin by CMC and that by Spinelli and Koury (1970) on the precipitation of sarcoplasmatic fish proteins by various phosphates.

MATERIALS AND METHODS

Preparation of defatted rapeseed meal

Rapeseeds (*Brassica napus*, winter variety Panter, obtained from AB Karlshamns Oljefabriker) were conditioned at room temperature to a water content of 8% and then subjected to a heat treatment for 18 minutes at 90 °C in an externally heated rotating drum. The seeds were crushed on a roller mill and the oil was removed by hexane extraction in a Soxhlet apparatus. Solvent was removed from the extracted flakes without heating and the flakes then were disintegrated in a hammer mil (Kamas SKB 200). The nitrogen content of the meal (as calculated on an oven dry basis) was 6.9 per cent.

Protein extraction

The meal was dispersed in deionized water at room temperature. The pH of the slurry was increased to 11.1 by the addition of aqueous sodium hydroxide. The extraction was allowed to proceed with stirring at constant pH for 30 minutes, which required small successive addition of aqueous sodium hydroxide. The final solid to liquid ratio was approximately 1/20 (w/w). Insoluble material was removed by centrifuging the dispersion twice at 3 100 x g for five minutes (Wifug X1 with swing-out rotor). By this extraction procedure 83% of the nitrogen content of the meal went into solution.

Precipitation

To the clarified protein extract was added, with thorough stirring, a solution of the precipitation aid (see below) to be studied. With the use of a peristaltic pump, 0.1 M hydrochloric acid was added to the mixture (50-70 ml) at a rate of 6 ml/min until the predetermined pH was reached. The dispersion was centrifuged for five minutes at 3 100 x g (Wifug X1 with swing-out rotor). The sediment was weighed and its dry matter content (DMC) determined after drying at 85 ^{o} overnight. The nitrogen content of the dried isolate was determined by the Kjeldahl technique. Protein contents were calculated as 5.5 times the nitrogen content (Tkachuk, 1969). The nitrogen yield was calculated as: (total nitrogen in the precipitate)/(total nitrogen in the extract from which the precipitate was prepared).

Phosphorus was determined according to Chen et al. (1956). The amount of phosphorus precipitated was determined as: (total phosphorus in the solution from which the precipitate was prepared) - (total phosphorus in the supernatant obtained after precipitation). The total amount of phosphorus in the supernatant was determined as: (concentration of phosphorus in the supernatant) x (the total volume of the protein extract, precipitation aid and acid added).

The following substances were used as precipitation aids: HMP, sodium hexametaphosphate (BDH) in the form of a freshly prepared 2% aqueous solution; CMC, carboxymethyl cellulose (kindly supplied by SCA, Sweden) of various DS and DP (DP = Degree of Polymerization; the average number of monomeric units by which a polymer is built. DS = Degree of Substitution; the average number of carboxyl groups per anhydroglucose unit), some of which were specially prepared for this investigation; sodium polygalactouronic acid (Eastman) or sodium alginate (Fluka) as a 1% aqueous solutions and carrageenans (lambda-, iota-, kappa-,) (kindly supplied by the Copenhagen Pectin Factory Ltd, Denmark) in the form of 0.5% aqueous solutions.

RESULTS AND DISCUSSION

Sodium hexametaphosphate (HMP) as a precipitation aid

The effect of sodium hexametaphosphate on the precipitation of rapeseed proteins are shown in Figure 1. The data derive from precipitation experiments on aliquot samples taken from a solution prepared by adding HMP to a rapeseed meal extract. As can be seen, addition of HMP resulted in a most pronounced narrowing of the precipitation maximum and brought about a large increase in the nitrogen yield. It also had a strong effect on the dry matter content (DMC) of the precipitate. In these respects, the results were similar to those obtained in a previously reported study on the effect of sodium phytate (sodium inositol hexaphosphate) on the precipitation of rapeseed protein extracts (Gillberg and Törnell, 1975).

In an experiment carried out under the same conditions as those used in the experiments shown in Figure 1, Gillberg and Lönnerdal (1975) have shown that only trace amounts of protein could be detected in the supernatant obtained after precipitation to pH 4.9. In their experiments the supernatant was subjected to gel filtration. The fractions containing high molecular weight substances showed only a very small absorption at 280 nm. Hence, there are reasons to believe that the maximum in the precipitation curve in Figure 1 corresponded to an almost quantitative recovery of the proteins.

The precipitation curves for nitrogen and phosphorus in Figure 1 have rather similar shapes, and it is evident that the effect exerted by the hexametaphosphate was due to its forming of co-precipitates with the proteins. A calculation based on the results presented showed that the P/N-ratio of the precipitates increased almost linearly with a decrease in the pH of precipitation. At pH 6.0 the P/N-ratio was 0.045 and at pH 3.0 it was 0.063 moles/mole. These values are very similar to the P/N-ratio as determined on the original rapeseed meal, which was found to be 0.050 moles/mole. The increase in the P/N-ratio with a decrease in pH is in agreement with the expectation assuming the reactions between the polyphosphate and the proteins are mainly governed by electrostatic interactions and that the precipitation depends upon mutual charge neutralization (cf. Smith and Rackis, 1957; Gillberg and Törnell, 1974). With decreasing pH, the number of free positive charges on the proteins increases, and thereby also the capacity of the proteins to react with the polyphosphate.

In the experiments in Figure 1, the maximum recovery of phosphorus was 82 per cent. Sodium hexametaphosphate corresponded to 77 per cent of the total phosphorus present, the remaining part originating from the rapeseed meal. These facts indicate that, under the conditions used, linear polyphosphates are effective complexing agents for rapeseed proteins.

As can be seen from Figure 1, the nitrogen yield decreased rapidly as the pH of precipitation fell below about 4. In this pH range, the number of negative charges on the protein (due to neutralization of carboxyl groups on asparagine and glutamine residues) decreases rapidly with a decrease in pH. If the capacity of the precipitation aid to form precipitates with the protein is already fully utilized, a decrease in pH should result in the formation of positively charged soluble complexes, and, as observed, in a sharp drop in the nitrogen yield.

Figure 2 shows the effects of the addition of different amounts of HMP to an alkaline meal extract, followed by a pH-adjustment to pH 4.9. In agreement with the results given in Figure 1, it can be seen that the nitrogen yield could be more than doubled by the addition of HMP. The very high nitrogen yield obtained at high additions of HMP must be ascribed to the co-precipitation of a large proportion of the non-protein nitrogen containing substances in the extract. A considerable amount of the nitrogen content in rapeseeds, 25-28%, are reported to be of this type (Miller et al., 1962). The results in Figure 2 also show the strong influence of HMP on the DMC of the protein isolates. This effect of HMP is favourable as a high DMC simplifies the separation of the product from the supernatant and also simplifies subsequent washing operations and the drying of the product.

According to Figure 2, the protein content of the isolates increased upon addition of moderate amounts of HMP. This result can imply precipitation, at moderate additions of HMP, of the strongly basic low molecular weight rapeseed proteins, which have a high nitrogen content. Another possible explanation is that HMP prevents other negatively charged seed components with a lower charge density (e.g. acid polysaccharides) from taking part in the precipitation of the proteins, thereby increasing the nitrogen content of the precipitates by reducing its non-protein content.

In the experiments of Figure 2, the amount of phosphorus in the supernatants and the P/N-ratio of the precipitates were determined. These results are shown in Figure 3. At additions of HMP up to the amount corresponding to the maximum in DMC (0.08 g HMP/g dissolved protein) (cf. Figure 2) the concentration of phosphorus in the supernatant was low and almost constant, indicating that most of the phosphorus added was bound to the precipitate. At larger additions, the concentration in the supernatant increased and, at the largest additions tested, the increase in the amount of phosphorus in the supernatant corresponded closely to the increase in the amount of phosphorus added. This is evident from the fact that at high additions the pertinent curve was almost linear, with a slope of unity. As can be seen by comparison with Figure 2, this occurred without a significant decrease in the nitrogen yield. These results imply that HMP, of the quality used and under the given conditions of pH and electrolyte concentration was unable to bring about a charge reversal of the proteins. This probably is a con-

sequence of the low DP of HMP, and may technically be a valuable property of HMP.

All of the present experiments have been carried out on extract solutions prepared at pH 11.1 and containing fairly low amounts of electrolytes. As has been previously shown in experiments at higher ionic strengths, much larger additions of HMP and quite different pH values were required in order to achieve high nitrogen yields (Gillberg and Törnell, 1974).

Carboxymethyl cellulose (CMC) as a precipitation aid

Results showing the influence of the pH of precipitation on the nitrogen yield and on the DMC of the precipitates at various additions of CMC are given in Figure 4. As can be seen, the larger the amount of CMC added, the narrower were the precipitation maxima. It can also be seen that the precipitation maxima were displaced towards lower pH values when the addition of CMC was increased. This displacement is in agreement with the assumption that the effect of CMC depends on its formation of neutral complexes with the proteins and that the complex formation involves the reaction between carboxyl groups on CMC and positively charged amino acid residues on the proteins. As has also been found in other systems (cf. Bungenberg de Jong, 1949; Gillberg and Törnell, 1974 and 1975) the maxima in the DMC occurred at slightly different pH values than the respective maxima in the nitrogen yield. According to Miller et al. (1962) 72-75% of the nitrogen content in rapeseed meal is of protein character. The high nitrogen yields obtained at the largest additions of CMC

indicate a co-precipitation of a large proportion of the non-protein nitrogen containing substances in the extract.

Figure 5 shows the effects of the addition of different amounts of CMC to an alkaline meal extract followed by a pH adjustment to pH 5.1. It is evident that at low additions, the effect of CMC on the precipitation was very similar to that of HMP (cf. Figure 2). In contrast to the behaviour of HMP, however, the nitrogen yield went through a very pronounced maximum as the addition of CMC was increased. This is a type of behaviour expected from any high molecular weight polyacid. Obviously, at additions of CMC beyond the maximum, the reaction between CMC and the proteins yielded negatively charged, soluble complexes. At the highest concentration of CMC tested, the nitrogen yield was much lower than that in the absence of CMC. This demonstrates the tendency of CMC to exert a protective colloid action. From the results given in Figure 5, it is evident that the use of CMC as a precipitation aid in the preparation of protein isolates requires a close control of the polymer dosage.

The maximum in Figure 5 occurred at a CMC concentration, which corresponded to an addition of 0.045 moles of carboxyl groups (in the form of CMC) per mole of nitrogen in the extract. This can be compared with the amount of phosphorus added as HMP in the experiments underlying Figure 1. This amount corresponded to 0.060 moles per mole of dissolved nitrogen. Thus, under the present conditions of pH and electrolyte concentration, CMC and HMP, when compared at equal concentrations of ionisable groups, seem to have about the same effectivity as precipitation aids.

The results in Figure 6 are from experiments with three samples of CMC, having about the same DP, but differing in DS, and those in Figure 7 are from experiments with CMC, differing in DP, but having almost equal DS. These experiments were carried out to compare the effectivity of the different CMC samples and were not extended to additions of CMC, where its protective colloid action was noticeable. The experimental data are too sparse to permit a discussion of the shape of the curves at high additions of CMC. It can be seen, however, that the effectivity, when compared on a weight basis, increased with an increase in DS (Figure 6). If instead, the effectivity is compared on a per carboxyl group basis, the order of effectivity is reversed, and the samples with the lowest DS are slightly more effective than the ones with the highest DS.

The results in Figure 7 imply, although small differences were obtained, that CMC with a high DP (high viscosity types) are not as effective as those with a low DP.

Polygalactouronic acid and alginates as precipitation aids

Experiments showed that these polymers behaved very similarly to CMC. Thus, when the precipitation was carried out in the presence of suitable amounts of these polymers, very high protein yields were obtained and the DMC of the precipitates was high.

Carrageenans as precipitation aids

The ionisable groups of the carrageenans are sulphate ester groups (Rees, 1969), in contrast to the other acidic polysaccharides tested which contain carboxyl groups. Results from precipitation experiments using iota-, kappa-, and lamda-carrageenans are given in Figure 8. Experiments were not carried out at polymer additions beyond the precipitation maxima, which means that the shape of the curves at high additions is uncertain. However, it can be seen, that the carrageenans are very effective as precipitation aids, kappa-carrageenan being the least effective. This is probably due to the fact that iota- and lambda-carrageenans have a higher content of sulphate groups than kappa-carrageenan.

An interesting feature, revealed by the results of Figure 8, was that the DMC of the precipitates was practically unaffected by the addition of carrageenan. In this respect, the carrageenans behave in a manner quite different from that of the other acidic polymers tested.

Influence of electrolytes

Electrostatic interactions play an important role in the precipitation of proteins both in the presence and in the absence of ionogenic precipitation aids. Accordingly, it is clear that the electrolyte composition of the protein extract must be an important parameter. For this reason, the influence of electrolytes on the precipitation of rapeseed proteins was studied. CMC was used as the precipitation aid.

The effect on the precipitation exerted by the presence of 0.1 M NaCl can be seen by comparing the broken and full lines in Figure 5. Except for the addition of NaCl, the conditions were the same in the two experimental series underlying this Figure. At all concentrations of CMC, the addition of sodium chloride resulted in very significant decreases in the nitrogen yield and in the dry matter content of the precipitates. The protein contents of the precipitates also decreased, but these decreases were comparatively small. In view of the fact that the protein extract used in the present experiments contained a large number of proteins with different isoelectric points, a detailed discussion of the results is not possible. It is quite clear, however, that as far as the influence of salt on the nitrogen yield and on the DMC is concerned, the effects observed are in good agreement with the effect of salt on the extent of complex coacervation and on the water content of the coacervate (Bungenberg de Jong, 1949). Addition of sodium chloride also resulted in a displacement of the maximum in nitrogen yield toward lower additions of carboxymethyl cellulose.

The fact that different salts may exert a different influence on the precipitation is shown by the results given in Figure 9 and 10. These results were obtained in experiments with rapeseed meal extracts containing various amounts of added salt and equal amounts of CMC (0.100 g CMC per g of protein). The pH of precipitation in all of the experiments was 5.1. As expected, divalent ions (cations as well as anions) had a greater effect

than monovalent ions (cf. Bungenberg de Jong, 1949). As can also be seen, large differences can exist in the effects exerted by ions of equal charge (Figure 10). The larger effect of chloride ions, as compared to acetate ions, can be explained by assuming chloride ions to be more strongly bound to the protein, an explanation in agreement with results from previous studies on selectivity in ion-protein interactions (cf. Norne et al., 1975).

REFERENCES

Barré, R.M. 1956. "Influence de l´acide phytique sur la digestion pepsique de différentes proteines". Ann. pharm. franc. 14:182.

Bungenberg de Jong, H.G. 1949. Complex colloid systems. In "Colloid science", Ed. Kruyt, H.R. Elsevier Publishing Company, The Netherlands.

Chen, P.S., Toribara, T.Y., Warner, H. 1956. Microdetermination of phosphorus. Analytical Chem. 28(11):1756.

O'Dell, B.L., Savage, J.E. 1960. Effect of phytic acid on zinc availability. Proc. Soc. Exp. Biol. and Med. 103:304.

Gillberg, L., Törnell B. 1974. Preparation of rapeseed protein isolates. Paper read at IV International Congress of Food Science and Technology, Madrid.

Gillberg, L., Törnell, B. 1975. Preparation of rapeseed protein isolates. I. Dissolution and precipitation of rapeseed proteins. J. Food Sci.

Gillberg, L., Lönnerdal, B. To be published.

Hidalgo, J., Hansen, P.M.T. 1969. Interaction between food stabilizers and β-lactoglobulin. J. Agri. Food Chem. 17(5):1089.

Miller, R.W., Van Etten, C.H., Mc Grew, C., Wolff, I.A., Jones, Q. 1962. Amino acid composition of seed meals from fortyone species of cruciferae. Agr. Food Chem. 10:426.

Morawetz, H., Hughes, W.L., Jr. 1952. The interaction of proteins with synthetic polyelectrolytes. I. Complexing of bovine serum albumin. J. Phys. Chem. 56:64.

Nitschmann, von Hs., Rickli, E., Kistler, P. 1959. "Ueber den Einfluss der Polyphosphate auf die Löslichkeit einiger Plasmaproteine und die Möglichkeit der Plasma-Fraktionierung mit Polyphosphat. Helv. Chim. Acta 42:2198.

Norne, J.-E., Hjalmarsson, S.-G., Lindman, B., Zeppezauer, M. 1975. Anion bindning properties of human serum albumin from halide ion quadropole relaxation. Biochem. In press.

Rees, D.A. 1969. Structure, conformation, and mechanism in the formation of polysaccharide gels and networks. Advances in Carbohydrate Chemistry and Biochemistry 24:279.

Reinhold, J.G., Nasr, K., Lahimgarzadeh, A., Heydayati, H. 1973. Effects of purified phytate and phytate-rich bread upon metabolism of zinc, calcium, phosphorus, and nitrogen in man. The Lancet 10:283.

Smith, A.K., Rackis, J.J. 1957. Phytin elimination in soybean protein isolation. J. Am. Chem. Soc. 79:633.

Spinelli, J., Koury, B. 1970. Phosphate complexes of soluble fish proteins. Their formation and possible uses. J. Agr. Food Chem. 18(2):284.

Tkachuk, R. 1969. Nitrogen-to-protein conversion factors for cereals and oilseed meals. Cereal Chem. 46:419.

Acknowledgements

Thanks are due to Mrs Jana Polackova for valuable experimental assistance, to Drs Ragnar Ohlson and Klas Anjou for providing us with rapeseed and for their continuous interest in this work and to Dr Axel Wennerblom for providing us with samples of CMC.

This work was made possible by grants from the Swedish Board for Technical Development. This financial support is gratefully acknowledged.

TEXT TO THE FIGURES

Figure 1. Influence of pH on the precipitation of a meal extract to which was added sodium hexametaphosphate (HMP) in an amount of 0.08 g per g protein.

0 Nitrogen yield

x Phosphorus recovery

□ DMC of the precipitates

The dotted curves represent an experiment carried out without addition of HMP.

Figure 2. Influence of the addition of HMP to a meal extract on the precipitation at pH 4.9 of the mixture. In this and all following figures, the addition of a precipitation aid is reported as gram precipitation aid (on an oven dry basis) per gram protein in the extract.

0 Nitrogen yield

□ Nitrogen content (N x 5.5) of the precipitates

Δ DMC of the precipitates.

Figure 3. Influence of the addition of HMP to a meal extract on the precipitation at pH 4.9 of the extract. The upper scale represents the total amount of P in the extract.

Δ The relative amount of phosphorus in the precipitates

0 The amount of phosphorus in the supernatant (g P per g protein in the original extract).

Figure 4. Influence of pH on the precipitation of a meal extract to which was added different amount of CMC (Cellugel 3 000 special).

	CMC/protein
x	0.025
0	0.062
□	0.098
Δ	0.166

Figure 5. Influence of the addition of CMC (Cellugel 3 000 special) to a meal extract on the precipitation at pH 5.1 of the mixture. The broken curves represent results obtained from experiments carried out in the presence of 0.1 M NaCl.

o Nitrogen yield

□ Protein content (N x 5.5)of the precipitates

Δ DMC of the precipitates.

Figure 6. Influence of the addition of CMC of different DS to a meal extract on the precipitation at pH 5.1 of the mixture.

	CMC	
	DS	DP
x	1.12	1100
□	0.77	1200
0	0.57	1200

Figure 7. Influence of the addition of CMC of different DP to a meal extract on the precipitation at pH 5.1 of the mixture.

	CMC	
	DS	DP
x	0.60	680
□	0.61	900
0	0.57	1200

Figure 8. Influence of the addition of carrageenan to a meal extract on the precipitation at pH 5.3 of the mixture.

0	lambda-
□	iota-
x	kappa-

Figure 9. The effect of adding a salt on the precipitation at pH 5.1 of a mixture of a meal extract and CMC (Cellugel 3 000 special, 0.10 g CMC/g protein): the effect of varying the cation using acetate as the anion.

□	NaAc
0	$CaAc_2$

Figure 10. The effect of adding a salt on the precipitation at pH 5.1 of a mixture of a meal extract and CMC (Cellugel 3 000 special, 0.10 g CMC/g protein): the effect of varying the anion concentration using sodium as the cation.

□	NaAc
0	NaCl
Δ	Na_2SO_4

FIGURE 1

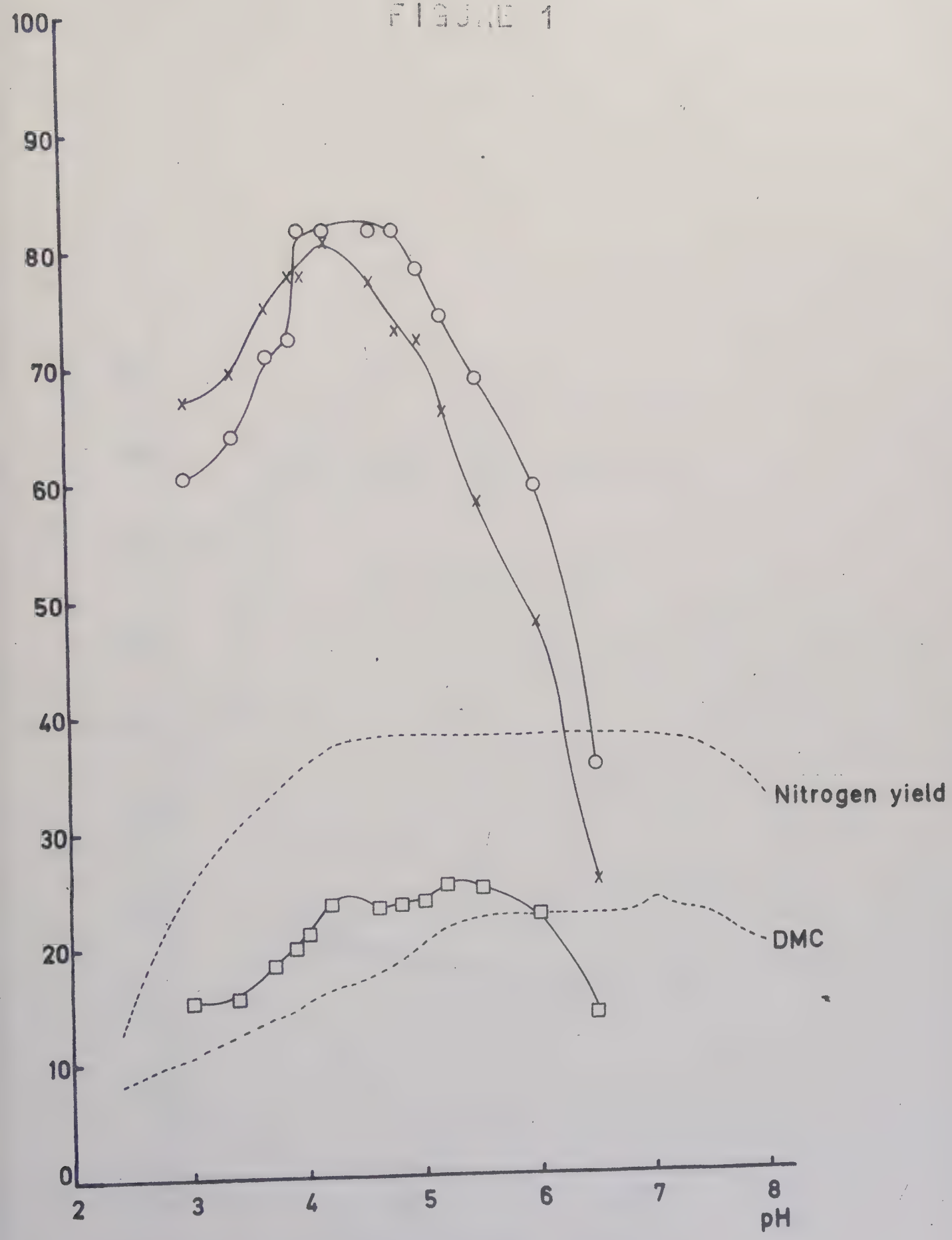


FIGURE 2

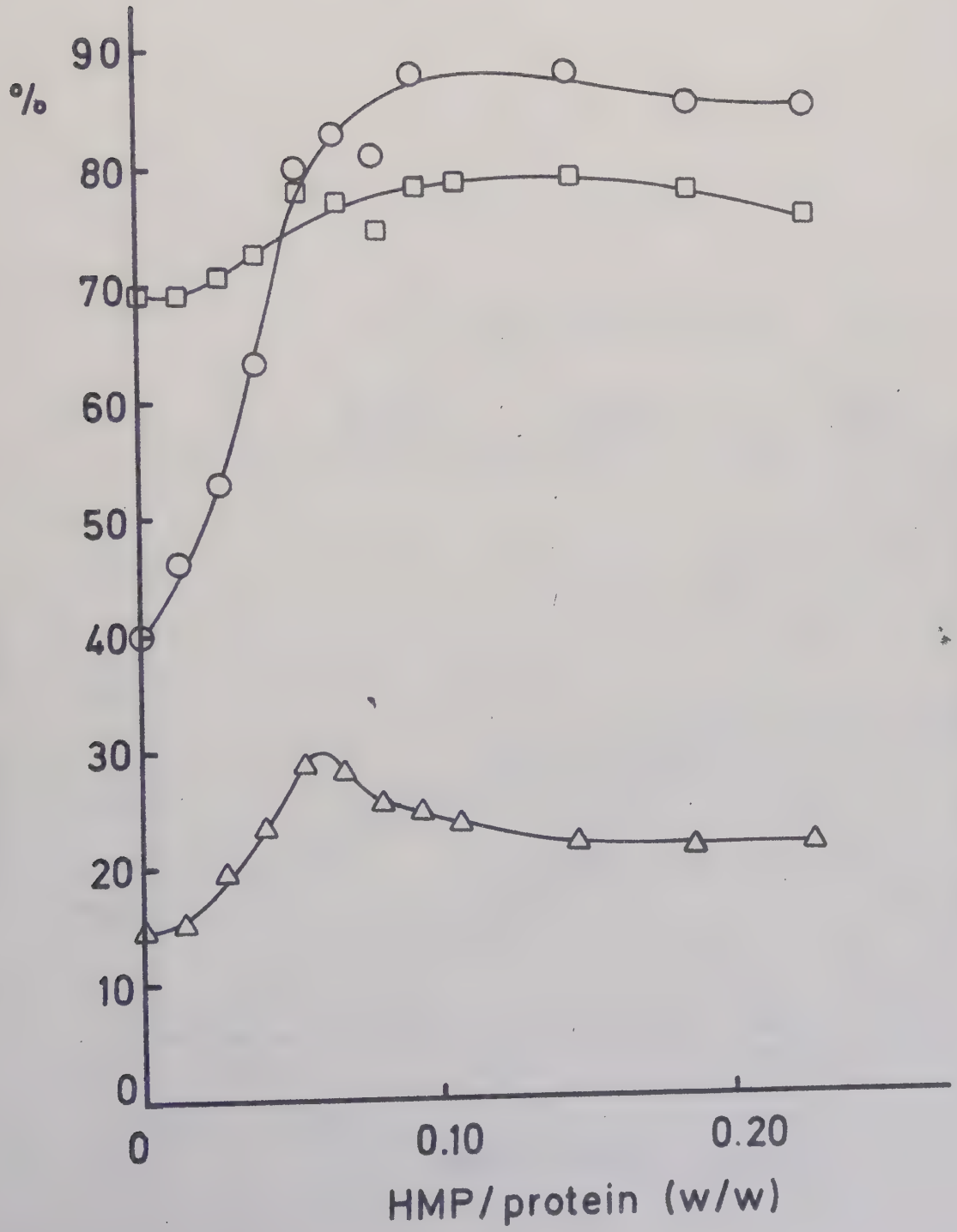


FIGURE 3

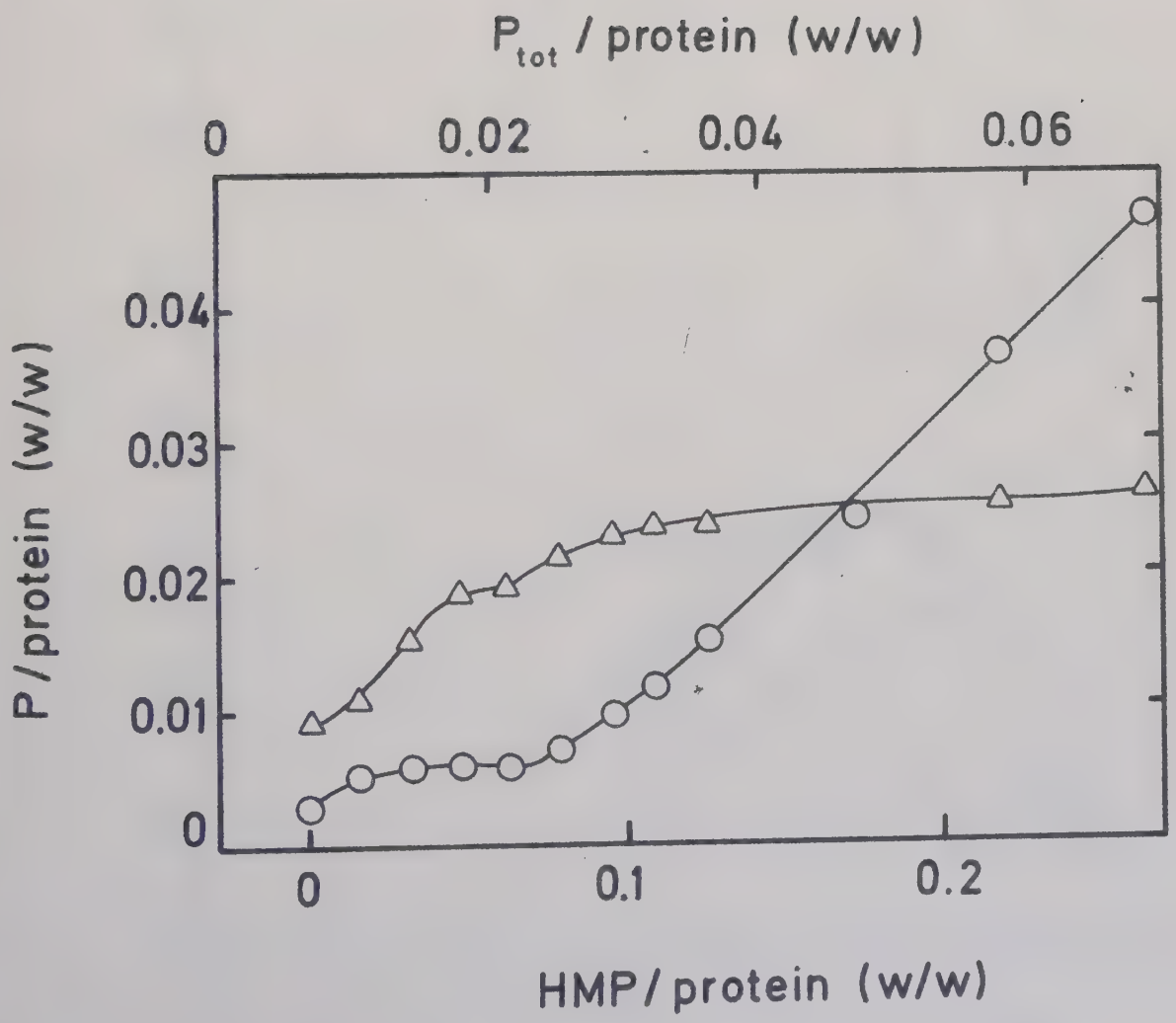

P_{tot} / protein (w/w)
0
0.02
0.04
0.06
0.04
0.03
0.02
0.01
0
P / protein (w/w)
0
0.1
0.2
HMP / protein (w/w)

FIGURE 4

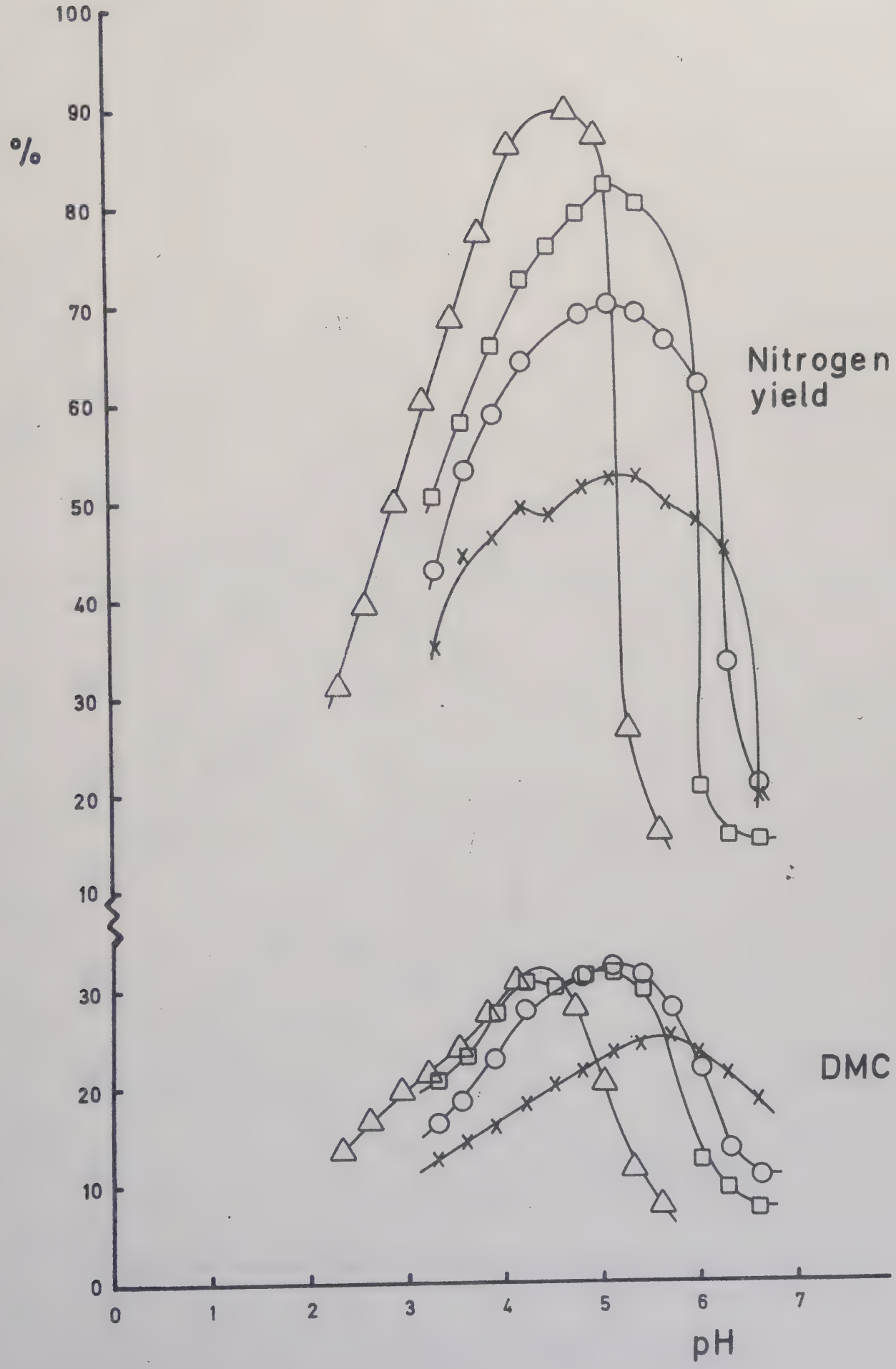

100
90
%
80
70
60
50
40
30
20
10
30
20
10
0
0
1
2
3
4
5
6
7
pH
Nitrogen yield
DMC

FIGURE 5

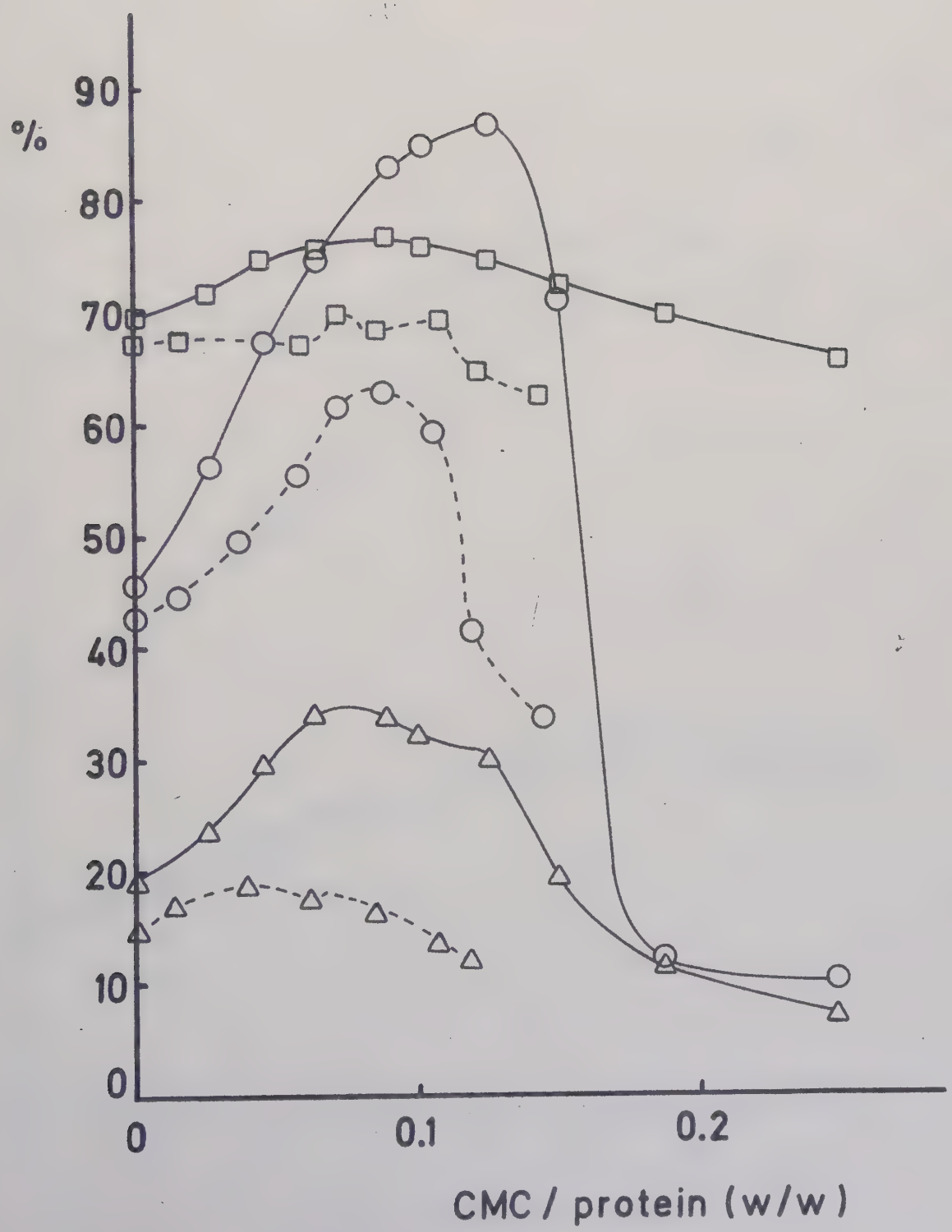


FIGURE 6

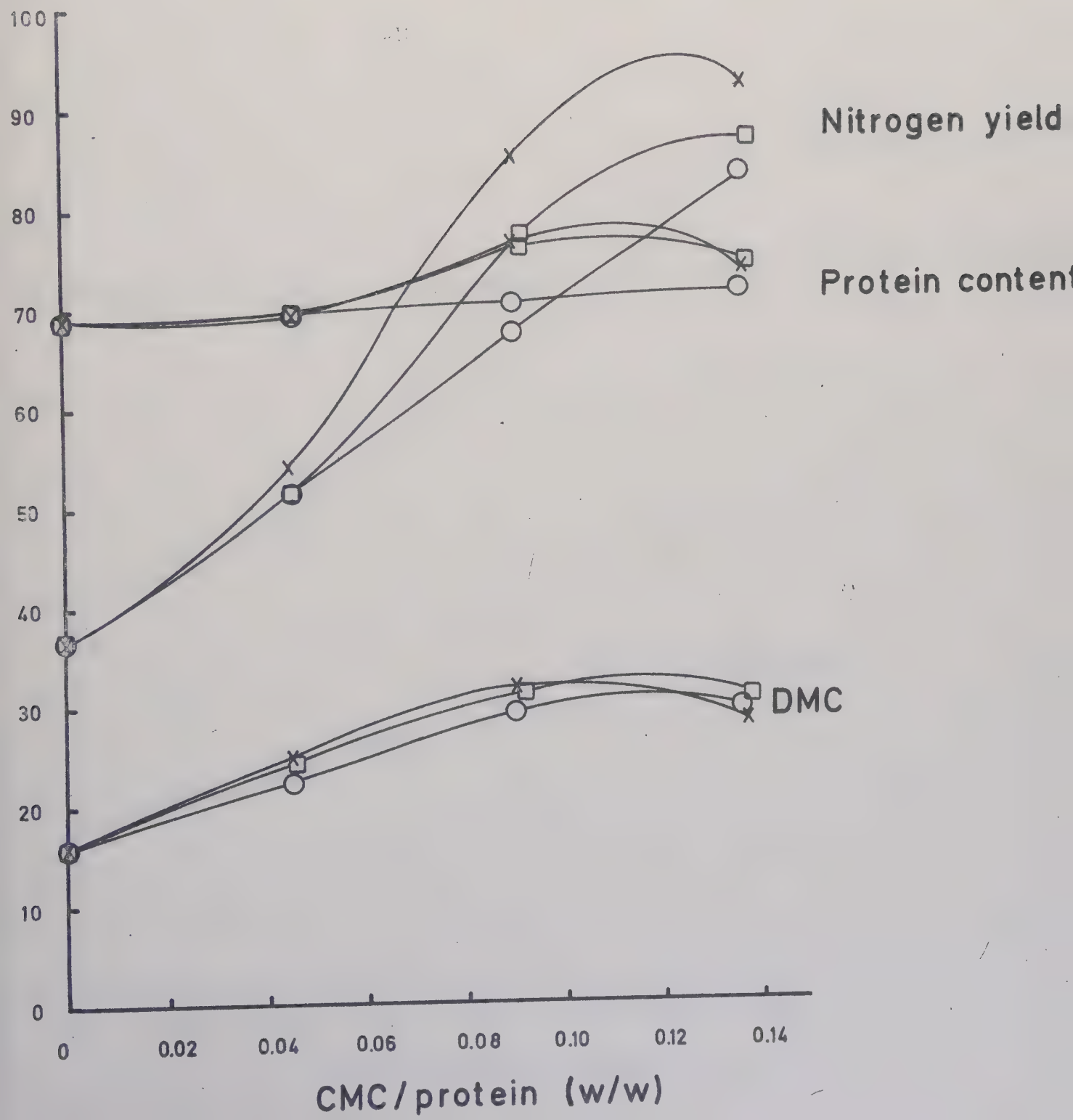

100
90
80
70
60
50
40
30
20
10
0
0
0.02
0.04
0.06
0.08
0.10
0.12
0.14
CMC/protein (w/w)
Nitrogen yield
Protein content
DMC

FIGURE 7

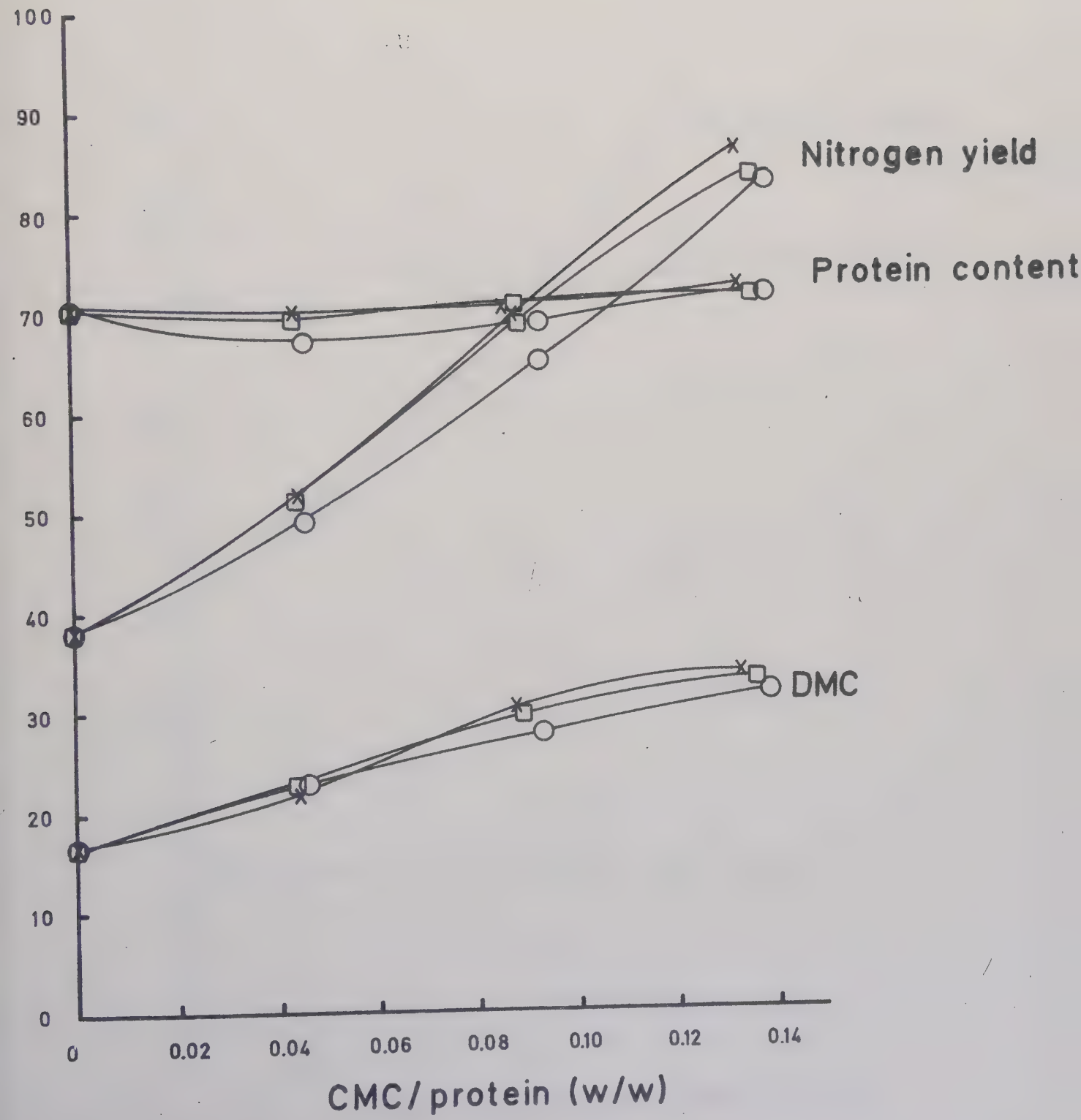

100
90
80
70
60
50
40
30
20
10
0
0
0.02
0.04
0.06
0.08
0.10
0.12
0.14
CMC/protein (w/w)
Nitrogen yield
Protein content
DMC

FIGURE 8

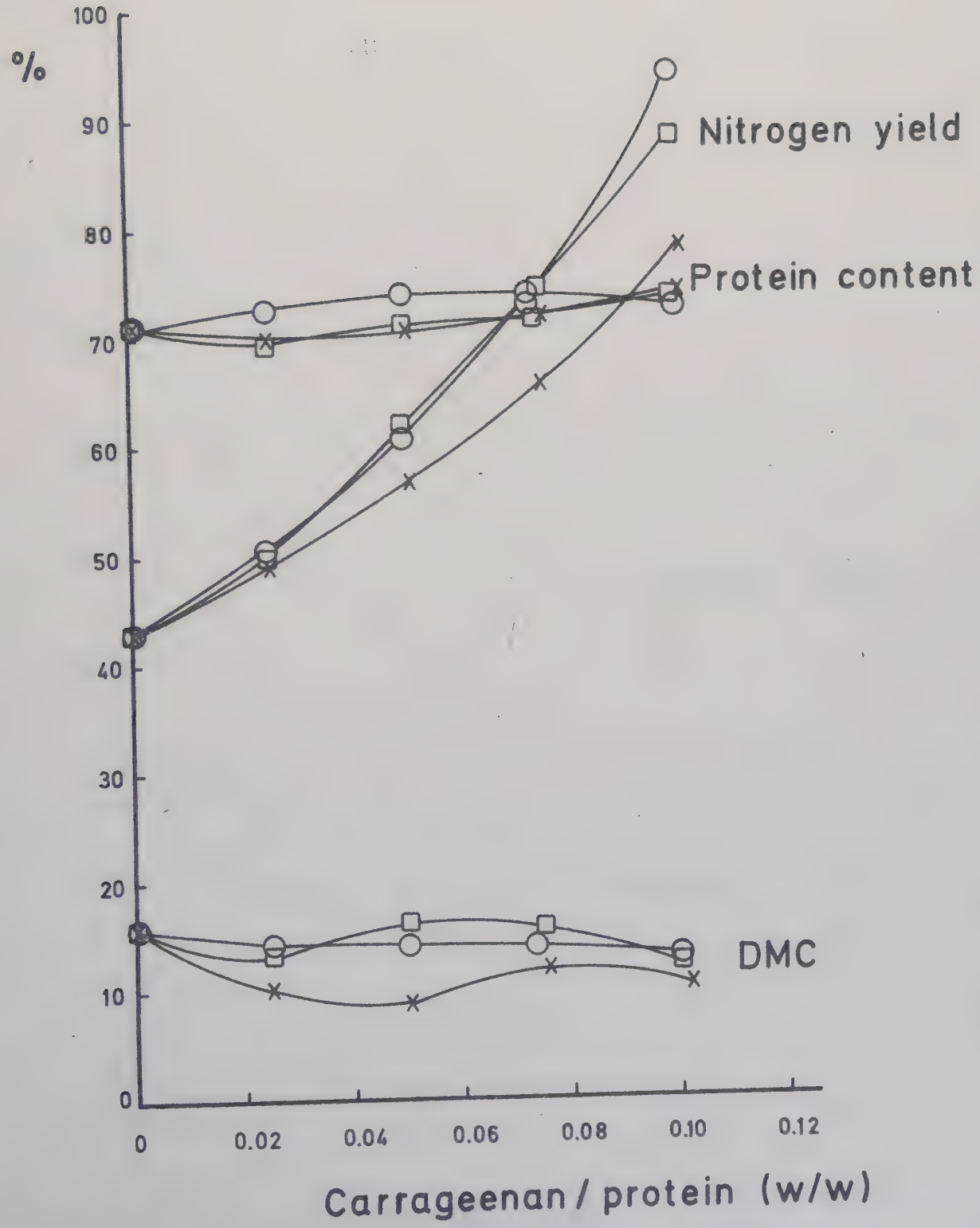

%
100
90
80
70
60
50
40
30
20
10
0
Nitrogen yield
Protein content
DMC
0
0.02
0.04
0.06
0.08
0.10
0.12
Carrageenan / protein (w/w)

FIGURE 9

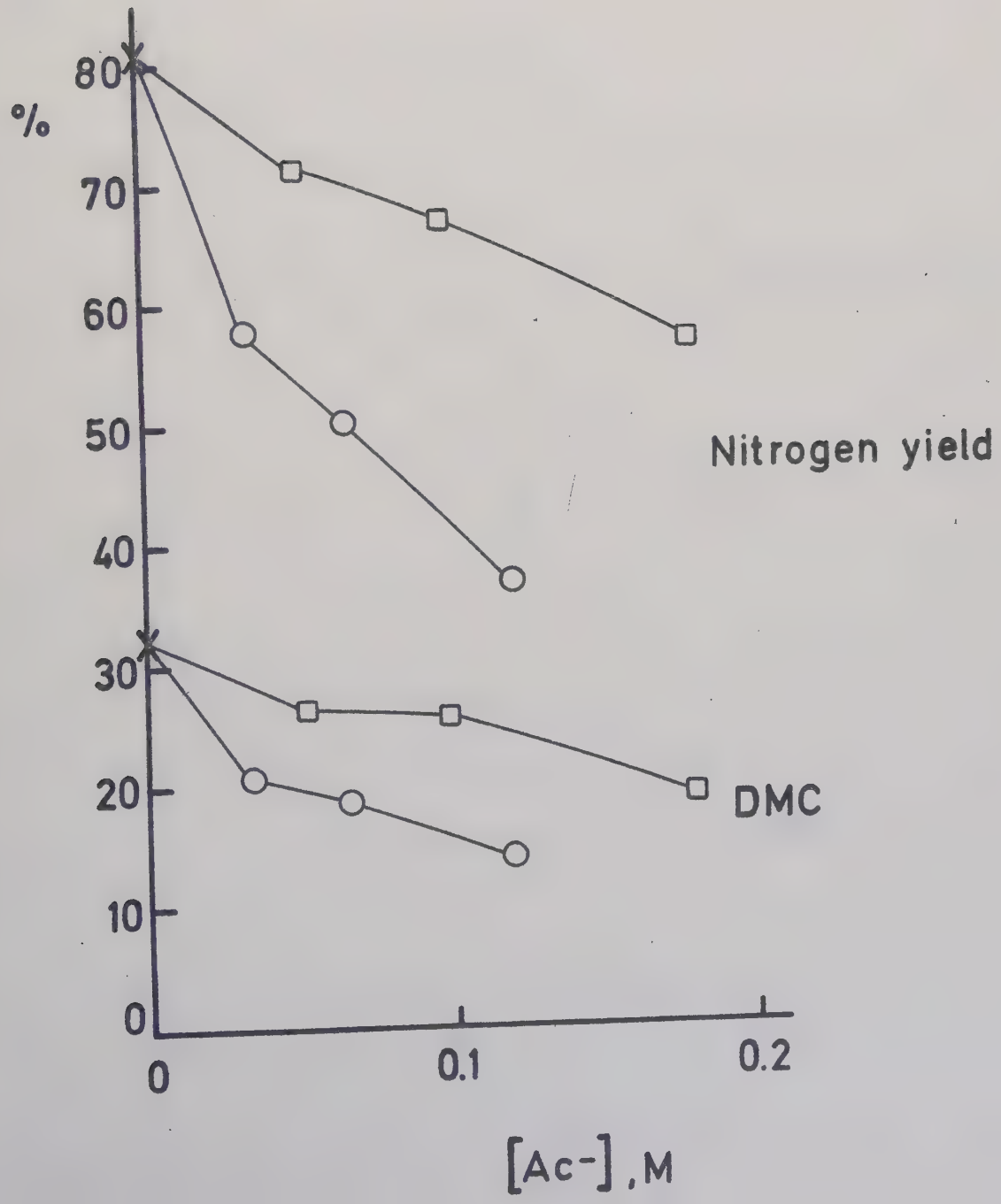
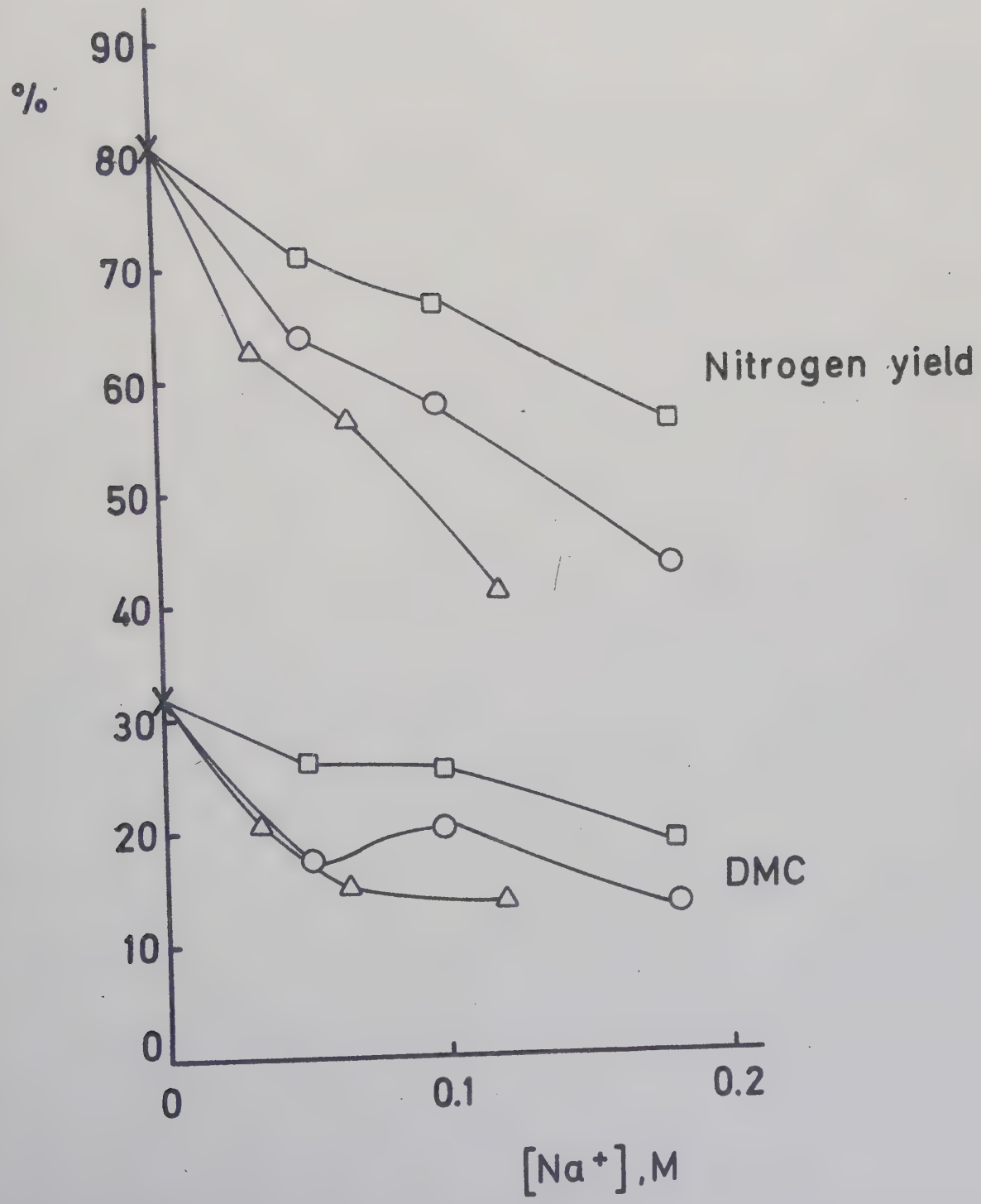


FIGURE 10



PREPARATION OF RAPESEED PROTEIN ISOLATES

III. A study of rapeseed protein isolates by molecular sieve chromatography.

Bo Lönnnerdal [1, x)], Lars Gillberg [2] and Bertil Törnell [2]

1. Institute of Biochemistry
   University of Uppsala
   P.O.B. 551
   S - 751 22 Uppsala
   Sweden

2. The Lund Institute of Technology
   Department of Chemical Technology
   P.O.B. 740
   S - 220 07 Lund 7
   Sweden

x) Present address: Institute of Nutrition
University of Uppsala
P.O.B. 551
S - 751 22 Uppsala
Sweden /

PREPARATION OF RAPESEED PROTEIN ISOLATES III. A study of protein isolates by molecular sieve chromatography.

B. Lönnerdal, L. Gillberg and B. Törnell. J. Food Sci.

Extraction of defatted rapeseed meal at alkaline pH followed by neutralization of the extract gives a protein isolate in a low yield. This material was found to be rich in acidic proteins and to contain all of the high molecular weight proteins. Quantitative recovery of the extracted proteins was achieved by adding an anionic polymer to the extract prior to acidification. Both methods were found to give protein isolates with a chemical score of 100. The isolates contained proteins with a variety of isoelectric points. A soluble complex between rapeseed proteins and CMC was shown to exist at pH 11.

INTRODUCTION

Rapeseed protein isolates can be prepared by extracting defatted rapeseed meal under alkaline conditions and then precipitating the dissolved proteins by acidification. This type of process, however, gives a rather low yield of isolate (Gillberg and Törnell, 1976 a). The low yield may be a consequence of the fact that rapeseed contains proteins with a wide range of molecular weights and isoelectric points (Lönnerdal and Janson, 1972 and 1973). As was recently shown (Gillberg and Törnell, 1976 b), the yield can be considerably increased if a suitable amount of an anionic polymer such as carboxymethyl cellulose (CMC) or sodium hexametaphosphate (HMP) is added to the extract prior to acidification.

In the present work, molecular sieve chromatography has been used to study the behavior of different protein and carbohydrate fractions during the preparation of protein isolates from an alkaline rapeseed meal extract. Amino acid analysis and isoelectric focusing have been used to further characterize the materials isolated.

MATERIALS AND METHODS

Preparation of protein isolates

Rapeseeds (*Brassica napus*, winter variety Panter) were obtained from AB Karlshamns Oljefabriker, Sweden. The seeds were heat treated, defatted and disintegrated, and the resulting meal extracted with aqueous sodium hydroxide at pH 11.1 as described

in a previous paper (Gillberg and Törnell, 1976 b). Three isolates, A, B and C, were prepared from the alkaline meal extract as shown schematically in Figure 1. The isolates were separated from their mother liquors by centrifugation for 5 minutes at 3 100 x g and lyophilized. Isolate A was prepared by adding 0.1 M HCl to the alkaline rapeseed meal extract to a pH of 6.6. Isolate B was prepared by adding 12 ml of a 1% aqueous solution of CMC (Cellugel 3000 Special, kindly supplied by SCA, Sweden) to 100 ml of supernatant A and adjusting the pH of the mixture to 5.0 by addition of 0.1 M HCl. Isolate C was prepared by adding 6 ml of a fresh 2% solution of HMP (BDH Chemicals Ltd, Poole, U.K.) to 100 ml of the alkaline meal extract and adjusting the pH of the mixture to 4.9. The amounts of HMP and CMC used were chosen to give a high nitrogen recovery.

Molecular sieve chromatography

The protein extract, the isolates and the supernatants were analyzed by molecular sieve chromatography on Sephadex G-200 (AB Pharmacia, Uppsala, Sweden) equilibrated in 0.01 M ammonium acetate/ammonia buffer of pH 11.0 containing 0.1 M NaCl. The column (1.8 x 80.0 cm) was calibrated with blue dextran, human serum albumin (dimer and monomer), chymotrypsinogen A, low molecular weight proteins from rapeseed (Molecular weight 13 000) (Lönnerdal and Janson, 1972) and glucose. The absorbance at 280 nm was used for protein assay and the orcinol-H_2SO_4-method, described by Svennerholm (1956), for hexose assay. The sample size was 1.8 ml, the flow rate 14 ml/h and the fraction volume 3.6 ml.

The isolates were prepared for analysis by dispersing 30 mg in 2 ml of the buffer (pH 11.0). Undissolved material was removed by centrifugation and the supernatant was dialyzed against the buffer. The dialyzed solution was used as sample.

The protein composition of the isolates was studied also by molecular sieve chromatography in 6 M urea at pH 8.0 using a column (1.5 x 80.0 cm) packed with DVS-agarose. This gel, cross-linked with divinylsulfone, is very stable and thus allows high flow rates. It was prepared at the Institute of Biochemistry in Uppsala (Porath et al., 1975). Each of the isolates (15 mg) was dissolved completely in 1 ml buffer solution (6 M urea with 0.05 M tris-HCl, pH 8.0). The flow rate was 6 ml/h and the column was calibrated as described above for the Sephadex column.

Amino acid analysis

The isolates were hydrolyzed in 6 M HCl for 24 hours at 110 ^{o}C. The hydrolysates were analyzed by the method of Spackman et al. (1958) using a Durrum D-500 Amino Acid Analyzer equipped with a PDP-8/L computer.

Isoelectric focusing

Isoelectric focusing was performed on the isolates dissolved in urea. The electrofocusing was performed as described by Wrigley (1968). The concentration of acrylamide was 7.5% and 2.5% of the total acrylamide was bisacrylamide ($T_{7.5}$, $C_{2.5}$).

The pH range of the ampholytes was 3-10 (LKB-Produkter, Stockholm, Sweden). The anode solution contained 0.2% H_2SO_4 in 6 M urea and the cathode solution consisted of 0.05 M NaOH in 6 M urea. The isolates (3 mg/ml) were dissolved in 6 M urea and a sample volume of 500 or 1 000 µl was used. The experimental runs were made at 4 °C for 16 hours. The proteins were precipitated with 5% trichloracetic acid (TCA) and the gels washed several times to remove the ampholytes. The washed gels were stained with 0.1% Amido Black for 5 hours and washed in 7% HAc. The gels were scanned at 540 nm with a Zeiss PMQ II spectrophotometer, equipped with an automatic scanning device. According to Bhatty (1972) rapeseed proteins, in contrast to most other proteins, are relatively soluble in 5% TCA. Separate experiments have shown, that it is the low molecular weight proteins with isoelectric points around 11, which are soluble in 5% TCA. These proteins should have moved into the anode solution and would consequently not be present in the gel.

RESULTS AND DISCUSSION

Figure 2 shows the molecular sieve chromatogram obtained for the alkaline meal extract on the Sephadex column. Two distinct protein fractions, corresponding to the peaks at fraction 20 and 27 can be distinguished. Their molecular weights were calculated to be over 250 000 and around 150 000 respectively. This is in agreement with the findings of Bhatty et al. (1968). The third rather broad protein peak corresponds to two groups of proteins, one having molecular weights of about 50-75 000 and the other having molecular weights around 13 000. (Lönnerdal and Janson, 1972; Lönnerdal, 1976). This last group has previously been se-

isolate B contained proteins of the low and intermediate molecular weight ranges only. This shows that precipitation of the alkaline rapeseed meal extract with hydrochloric acid, as in the preparation of isolate A, resulted in a total precipitation of the high molecular weight proteins.

Figure 4 represents molecular sieve chromatograms of samples obtained by dispersing the isolates in the pH 11 buffer. It should be noted that the isolates did not dissolve completely in the buffer. The fact that only small amounts of high molecular weight proteins occurred in the chromatogram in Figure 4 A, thus indicates that the high molecular weight proteins in isolate A did not easily redissolve at pH 11.

According to Figure 4 isolate B gave both a protein and carbohydrate peak at fraction 23, which would indicate the presence of high molecular weight proteins and carbohydrates. The carbohydrate peak at fraction 23 was probably due to the presence of CMC in the isolate. In a separate test CMC was found to give a peak at this position. The protein peak at fraction 23 in Figure 4 B is not consistent with the results of Figure 3, which clearly show that isolate B contained no high molecular weight proteins. This apparent inconsistency is most probably due to the formation of soluble complexes between the proteins and the CMC in the buffer used. Thus molecular sieve chromatography in different media may be a valuable tool for detecting the presence of such complexes.

Figure 5 shows molecular sieve chromatograms of supernatant A, B and C. As can be seen from Figure 5 supernatants B and C contained very little if any protein or other substances absorbing at 280 nm. This shows that rapeseed proteins can be quantitatively precipitated either in a one-step process or in a two-step process using CMC or HMP as a precipitation aid. Since other polymeric anions have been found to be as effective as CMC and HMP in improving the nitrogen recovery (Gillberg and Törnell, 1976 b), it is likely that most anionic polymers can be used for a quantitative precipitation of the proteins.

As seen from Figure 5 supernatant B contained no carbohydrates at fraction 25 and higher. This indicates that all added CMC was precipitated when isolate B was prepared.

Figure 6 shows a photograph of the gels used in the isoelectric focusing experiments. All of the isolates had a broad spectrum of isoelectric points. Isolate A was found to be especially rich in acidic proteins (Ip 4-6). This is consistent with the presence of large amounts of high molecular weight proteins in isolate A and the fact that these proteins are known to have isoelectric points in the range 4 to 7 (Ohlson and Sepp, 1975). Isolate B was found to be especially rich in neutral proteins (Ip 6-8). This finding is in a good agreement with the observation that isolate B only contained proteins with intermediate and low molecular weights. The intermediate molecular weight proteins are known to have isoelectric points in the range 5 to 8 (Ohlson and Sepp, 1975). The strongly basic low molecular weight proteins

did not show up in the isoelectric focusing experiments due to their high isoelectric points. Scanning of the gel used for isolate C (Figure 6) demonstrates the presence of proteins with a variety of isoelectric points. At least 19 bands with isoelectric points ranging from 4.5 to 10 can be seen.

Table 1 shows the amino acid composition of the isolates. The isolates had a very well balanced amino acid pattern, illustrated by their high chemical score, which for the A and C isolates were calculated to be 100. For isolate B lysine was the first limiting amino acid closely followed by tyrosine. The facts that the content of cystein was high and that no trace of lysinoalanin was found in the isolates, indicate that detrimental changes in the nutritive value of the proteins were not produced during the extraction of the rapeseed meal at pH 11.1.

Experiments similar to those discussed above, were carried out starting from a meal prepared from not previously heat treated rapeseed. The results obtained were approximately the same as those obtained from the meal from heat treated seeds. Thus it was found that all of the high molecular weight proteins precipitated upon acidification of the meal extract. This isolate was found to contain proteins with a variety of isoelectric points but was especially rich in acidic proteins. The remaining proteins could be precipitated by CMC giving a mother liquor free of proteins. Soluble complexes in the pH 11 buffer, made up of low and intermediate molecular weight proteins and CMC, were also formed with these isolates.

REFERENCES

Bhatty, R.S., Mc Kenzie, S.L. and Finlayson, A.J. 1968. The proteins of rapeseed soluble in salt solutions. Can. J. Ciochem. 46:1191.

Bhatty, R.S. 1972. A note on trichloroacetic acid precipitation of oilseed proteins. Cereal Chem. 49:729.

Gillberg, L. and Törnell, B. 1976 a. Preparation of rapeseed protein isolates I. Dissolution and precipitation behavior of rapeseed proteins. J. Food Sci. In press.

Gillberg, L. and Törnell, B. 1976 b. Preparation of rapeseed protein isolates II. Precipitation of rapeseed proteins in the presence of polyacids. J. Food Sci. In press.

Lönnerdal, B. and Janson, J.-C. 1972. Studies on Brassica seed proteins I. The low molecular weight proteins in rapeseed. Isolation and characterization. Biochim. Biophys. Acta 278:175.

Lönnerdal, B. and Janson, J.-C. 1973. Studies on myrosinases II. Purification and characterization of a myrosinase from rapeseed (Brassica napus L.). Biochim. Biophys. Acta 315:421.

Lönnerdal, B. 1976. To be published.

Ohlson, R. and Sepp, R. 1975. "Proteins and other crucifers" in Pirie, N.W. ed. Food protein sources. Cambridge University Press.

Porath, J., Låås, T. and Janson, J.-C. 1975. Agar derivatives for chromatography, electrophoresis and gel bound enzymes III. Rigid agarose gels cross-linked with divinyl sulfone (DVS). J. Chromatogr. 103:49.

Spackman, D.H., Stein, W.H. and Moore, S. 1958. Automatic recording apparatus for use in the chromatography of amino acids. Analyt. Chem. 30:1190.

Svennerholm, L. 1956. The quantitative estimation of cerebrosides in nervous tissue. J. Neurochem. 1:42.

Wrigley, C. 1968. Gel electrofocusing - a technique for analysing multiple proteins samples by isoelectric focusing. Sci. Tools 15(2):17.

ACKNOWLEDGEMENTS

We are grateful to Mrs Annika Jägare for skilful technical assistance. The Zeiss photometer with the scanning device was kindly put at our disposal by Professor S. Hjertén. Financial support given by the Swedish Board for Technical Development is gratefully acknowledged.

Table 1. Amino acid composition of the isolates (mg amino acid/g isolate on as is basis). The chemical score was calculated using the reference amino acid pattern given by FAO 1973.

Amino acid	Isolate		
	A	B	C
Arg	58.9	56.6	55.9
Lys	44.4	38.0	45.0
His	23.1	23.5	22.9
Asp	57.2	52.8	48.6
Glu	140.4	191.6	153.3
Cys	13.4	16.4	21.6
Met	17.9	17.3	18.1
Ile	36.2	32.3	33.6
Leu	75.0	63.7	60.7
Tyr	29.5	16.3	24.8
Phe	34.0	31.4	34.0
Pro	60.9	69.2	68.6
Gly	40.5	39.2	39.1
Ala	38.0	33.3	35.0
Ser	42.0	36.8	37.2
Thr	38.6	28.2	33.5
Val	43.7	39.8	40.6
NH_3	19.5	26.6	13.5
Chemical score	100	79	100
First limiting amino acid	---	Lys	---

Rapeseed protein isolates III.

TEXT TO THE FIGURES

Figure 1. Scheme for the preparation of protein isolates.

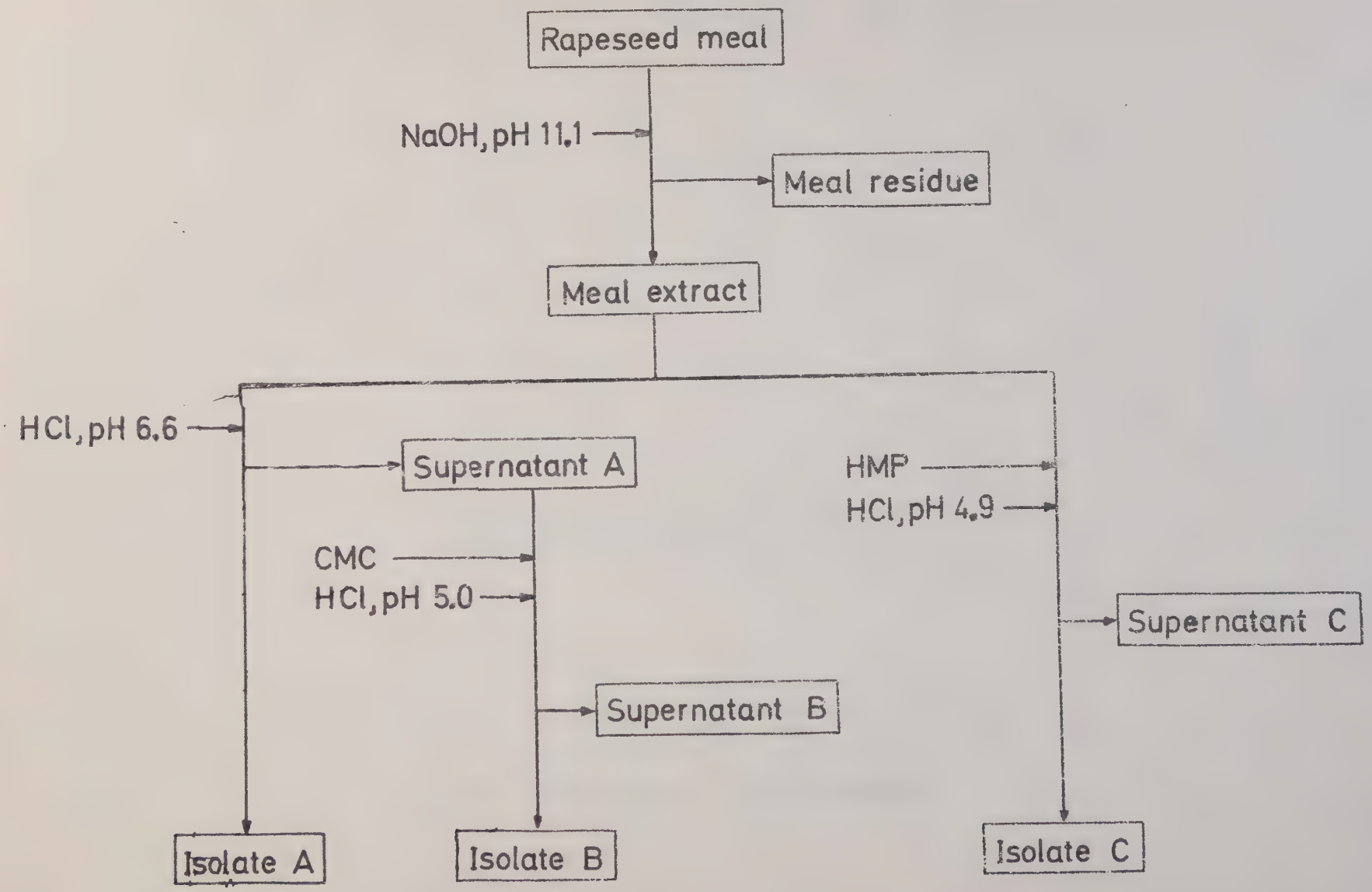
Figure 2. Molecular sieve chromatogram of the rapeseed meal extract on Sephadex G-200 in 0.01 M ammonium acetate/ammonia buffer of pH 11.0 containing 0.1 M NaCl.

Figure 3. Molecular sieve chromatogram of isolates A, B and C on DVS-agarose in 6 M urea with 0.05 M tris-HCl buffer of pH 8.

Figure 4. Molecular sieve chromatogram of isolates A, B and C on Sephadex G-200 in the pH 11.0 buffer.

Figure 5. Molecular sieve chromatogram of supernatants A, B and C on Sephadex G-200 in the pH 11.0 buffer.

Figure 6. Gels from the isoelectric focusing experiments with isolates A, B and C. The scanning trace is for the gel used for isolate C.


Rapeseed meal
NaOH, pH 11.1
Meal residue
Meal extract
HCl, pH 6.6
Supernatant A
HMP
HCl, pH 4.9
CMC
HCl, pH 5.0
Supernatant C
Supernatant B
Isolate A
Isolate B
Isolate C

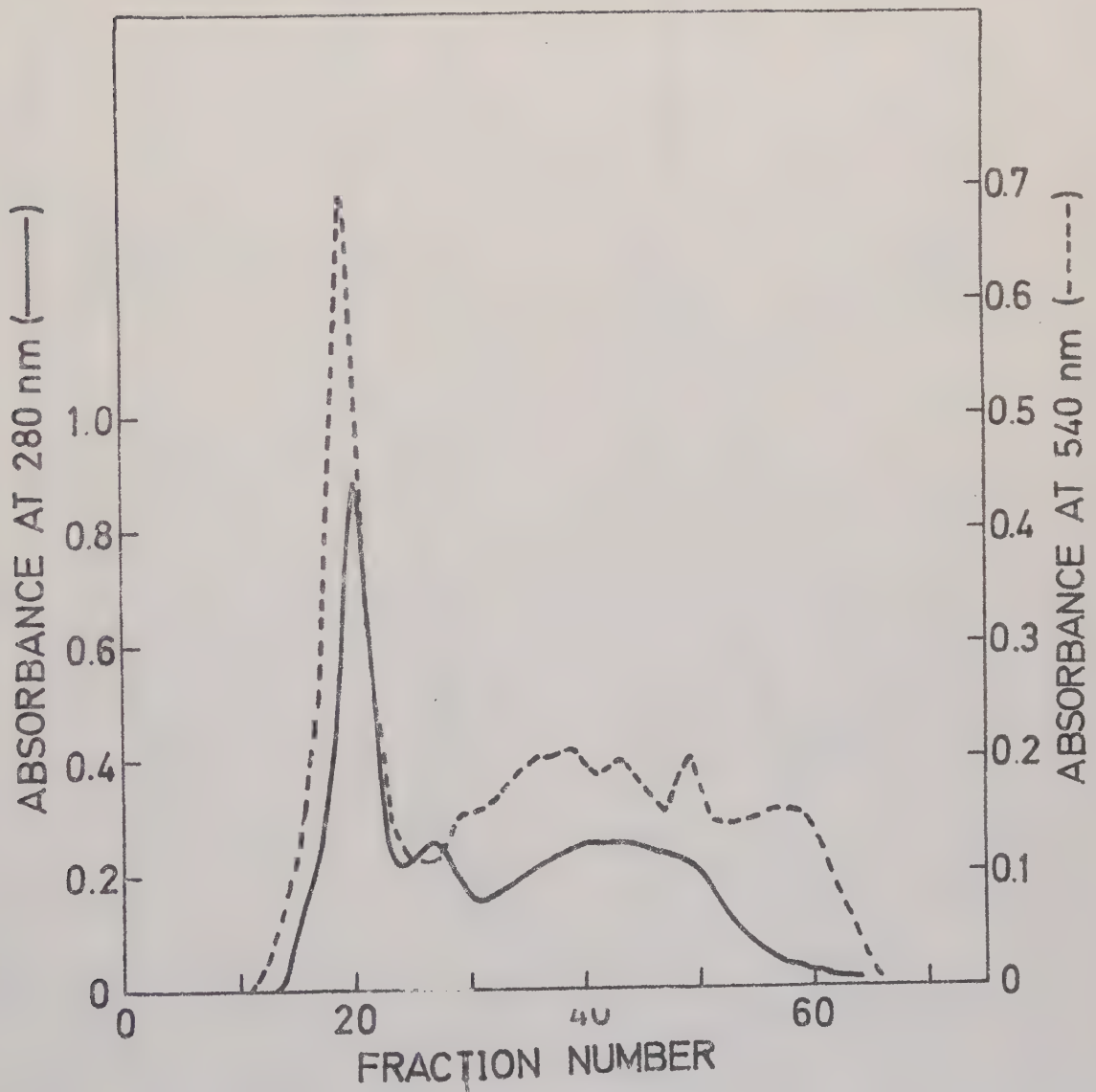

ABSORBANCE AT 280 nm (——)
ABSORBANCE AT 540 nm (- - - - -)
1.0
0.8
0.6
0.4
0.2
0
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0
20
40
60
FRACTION NUMBER

FIGURE 2

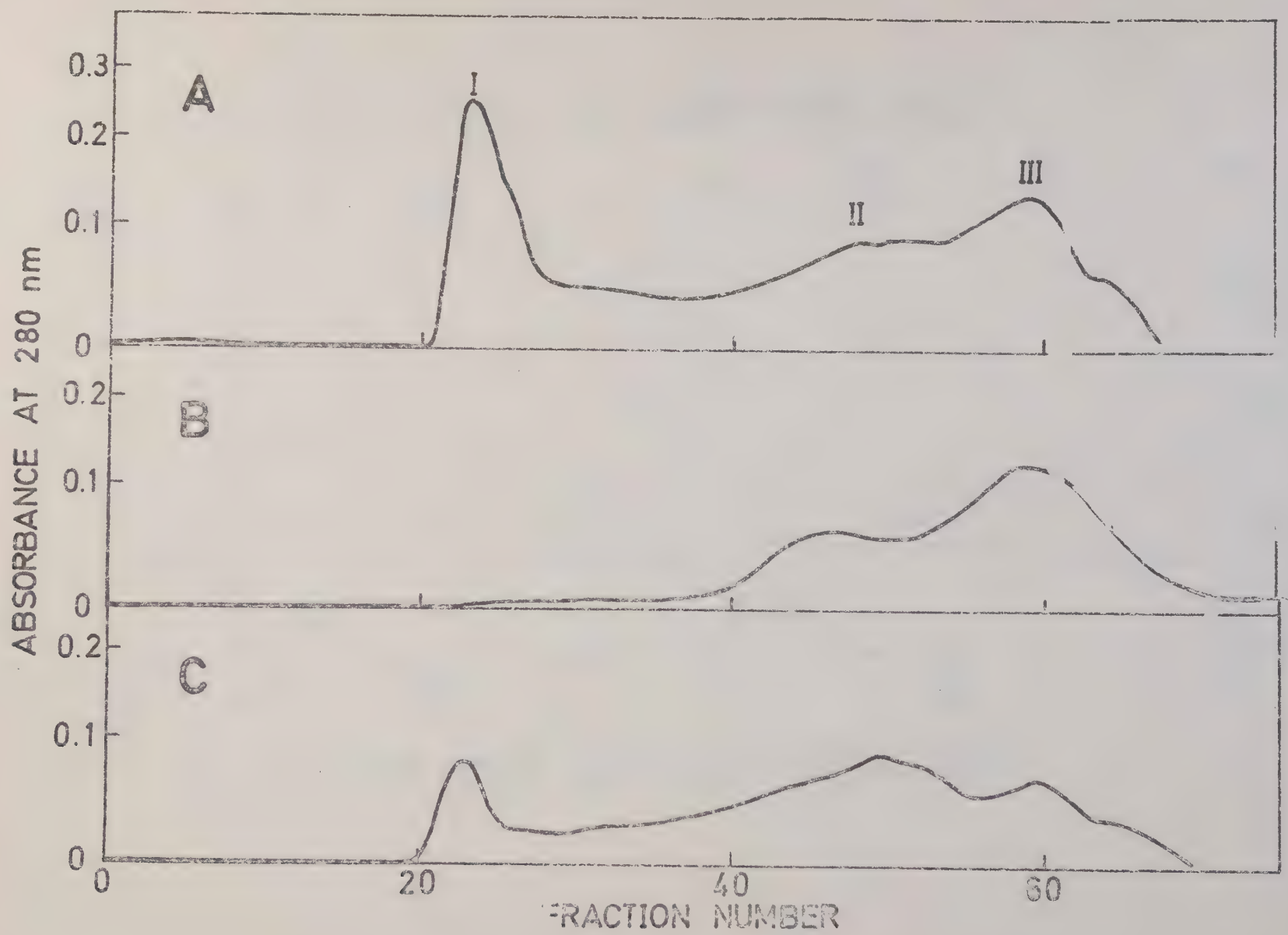

A
B
C
I
II
III
0.3
0.2
0.1
0
ABSORBANCE AT 280 nm
0
20
40
60
FRACTION NUMBER

FIGURE 3

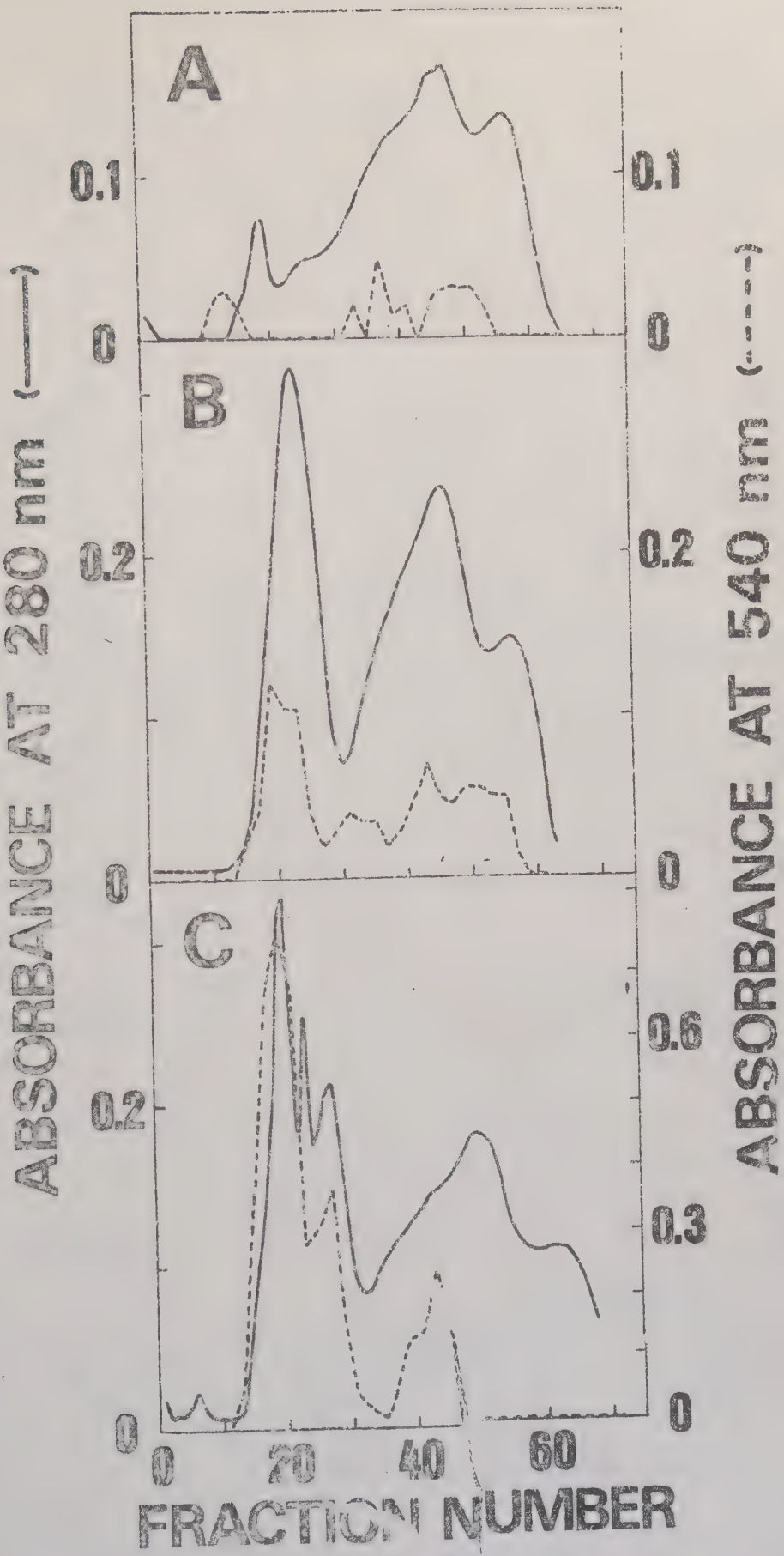

A
B
C
0.1
0
0.2
0
0.2
0
0.1
0
0.2
0
0.6
0.3
0
0
20
40
60
ABSORBANCE AT 280 nm (——)
ABSORBANCE AT 540 nm (- - - -)
FRACTION NUMBER

FIGURE 4

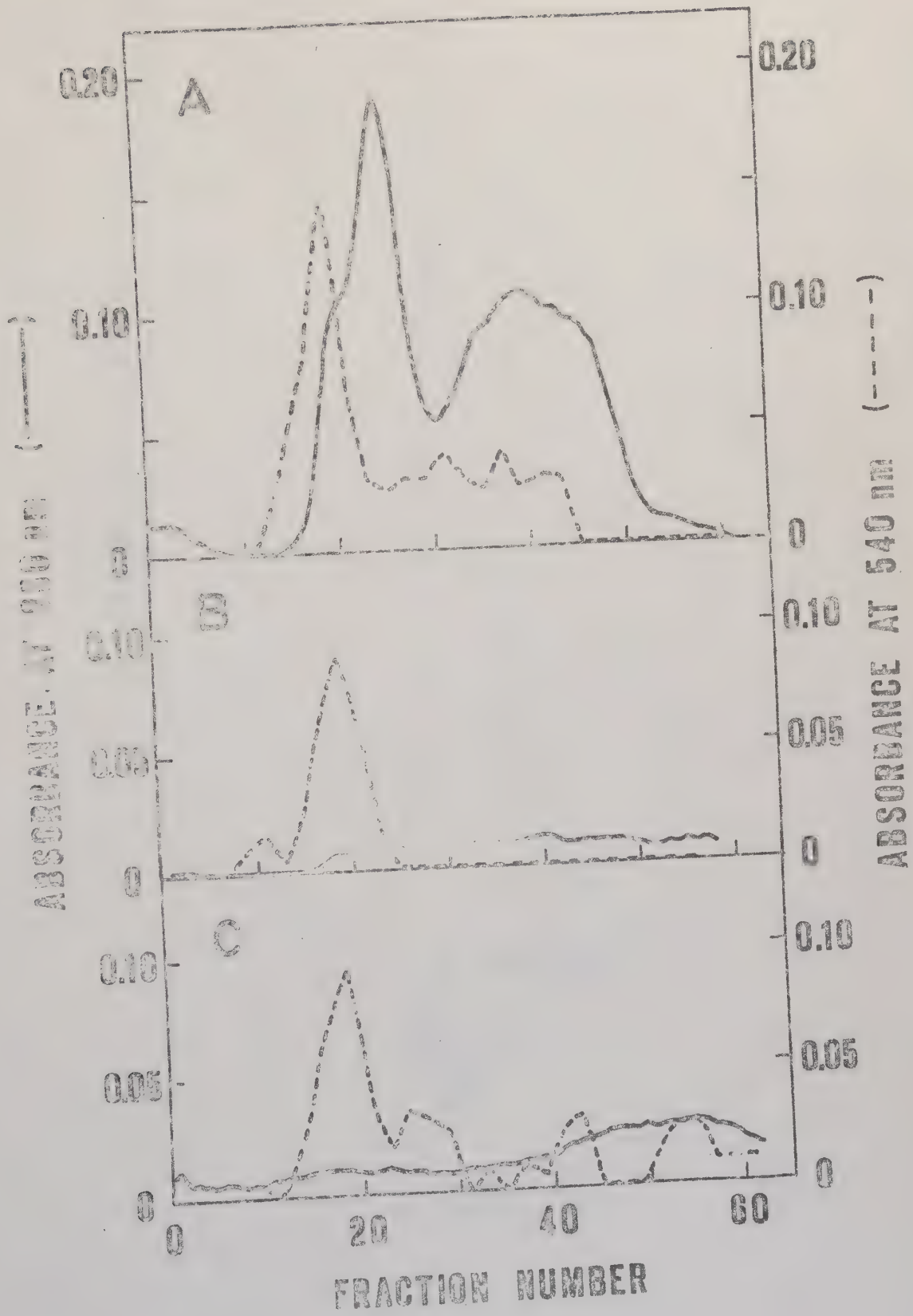

A
B
C
0.20
0.10
0
0.05
ABSORBANCE AT 540 nm (- - - -)
ABSORBANCE AT 280 nm (——)
0
20
40
60
FRACTION NUMBER

FIGURE 5

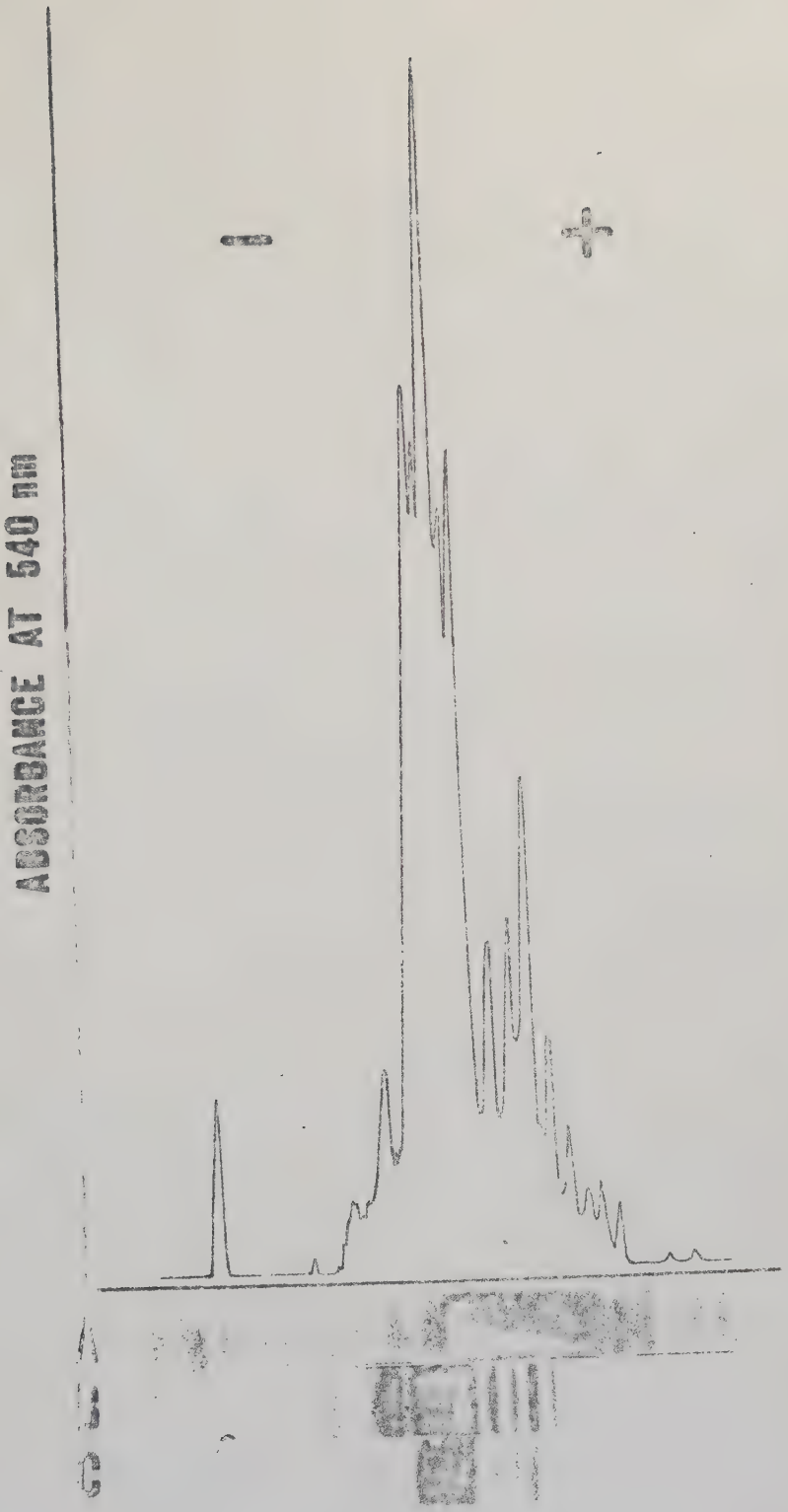


FIGURE 5

IV

PREPARATION OF RAPESEED PROTEIN ISOLATES

IV. A study of the distribution of carbohydrates in the preparation of rapeseed protein isolates.

Per Åman [1] and Lars Gillberg [2]

1. Department of Chemistry
   Agricultural College of Sweden
   S - 750 07 Uppsala 7
   Sweden

2. The Lund Institute of Technology
   Department of Chemical Technology
   P.O.B 740
   S - 220 07 Lund 7
   Sweden

Rapeseed protein isolates

PREPARATION OF RAPESEED PROTEIN ISOLATES. IV. A study of the distribution of carbohydrates in the preparation of rapeseed protein isolates. Per Åman and Lars Gillberg.

The distribution of carbohydrates in the preparation of rapeseed protein isolates was studied. The major proportion of the neutral carbohydrates of rapeseed meal was found to accumulate in the meal residue and supernatant. Only a minor amount was found in the isolates. The carbohydrates in the supernatant were mainly oligosaccharides. The acidic carbohydrates as well as the RNA extracted from the rapeseed meal were completely recovered in the protein isolate. It was suggested that protein isolates consist of products in which not only proteines but also polyacids (e.g. RNA, phytic acid, acidic polysaccharides and acidic polyphenols) extracted from the raw material are accumulated.

INTRODUCTION

Defatted and dehulled rapeseed (*Brassica napus*) and turnip rapeseed (*Brassica campestris*) meals contain about 10% low-molecular weight carbohydrates. The amount of neutral constituents of the polysaccharides in dehulled rapeseed meal accounts for about 12% of the dry meal (Theander and Åman, 1974). The low molecular weight carbohydrates in rapeseed have been studied by Siddiqui et al. (1973) and Theander and Åman (1976 a). In meal from *Brassica napus* and arabinan (Larm et al., 1975), a neutral arabinogalactan (Larm et al., 1976) and an alkali soluble amyloid (Theander and Åman, 1976 b) and in meal from *Brassica campestris* a water soluble amyloid (Siddiqui and Wood, 1971), an acidic arabinogalactan (Siddiqui and Wood, 1972) and an arabinan (Siddiqui et al., 1973) have been isolated and structurally investigated. The pectin in rapeseed hull has been studied by Aspinall and Jiang (1974) and the carbohydrates, polyphenols and lignin in the hulls by Theander et al. (1976).

Little is known about the distribution of the carbohydrates in protein isolate processes. An attempt to follow different carbohydrate fractions during the preparation of rapeseed protein isolates was made by Lönnerdal et al. (1976), using molecular sieve chromatography. This paper presents results from a study of the composition of carbohydrates in protein isolates prepared from an alkaline rapeseed meal extract. The meal residue and a supernatant were also studied.

EXPERIMENTAL

Preparation of protein isolates

Rapeseed (winter variety, Panter) was obtained from AB Karlshamns Oljefabriker, Sweden. The seeds were subjected to a heat treatment (inactivation of myrosinase), defatted, disintegrated and extracted at room temperature by aqueous sodium hydroxide at pH 11.1 as previously described (Gillberg and Törnell, 1976 b). The meal residue was neutralized to pH 7 and lyophilized. From the alkaline extract three protein isolates, A, B and C, were prepared as shown schematically in Figure 1. The isolates were recovered by centrifugation, washed three times with deionized water and lyophilized. Isolate A was obtained by adding a solution of 0.1 M HCl to the meal extract until a pH of 6.6 was reached. Isolate B was prepared by adding 0.12 g CMC, carboxymethyl cellulose (Cellugel 3 000 Special, kindly supplied by SCA, Sweden), as a 1% solution to 100 ml of supernatant A and adjusting the pH of the mixture to 5.0. Isolate C was prepared by adding 0.12 g HMP, sodium hexametaphosphate (BDH Chemicals Ltd, Poole, U.K.) as a 2% solution to 100 ml of the alkaline extract and then adjusting the pH to 4.9. By the above processes a quantitative precipitation of the proteins in the alkaline meal extract is obtained (Lönnerdal et al., 1976).

Analysis

All concentration steps in the analytical procedures were performed either at reduced pressure and bath temperatures not exceeding 40 °, or by freeze drying. All results were calculated

on a dry weight basis. Glucosinolates were determined according to Persson (1974) and phytic acid using the method of Wheeler and Ferrel (1971).

Paper chromatography and paper electrophoresis. Paper chromatography using the following systems (v/v): a) ethylacetate-acetic acid-water, 3:1:1 and b) pyridine-ethyl acetate-water, 2:8:1 and paper electrophoresis, using 0.5 M sodium acetate-acetic acid buffer (pH 4.5) at 1 500 V for 2 hrs, were run on Whatman No. 1 paper and the components were detected with *p*-anisidine hydrochloride or silver nitrate-sodium hydroxide.

Sugar analysis. To 100 mg samples of the isolates, the meal residue and the CMC 2.00 mg *myo*-inositol was added as internal standard. The samples were hydrolyzed (suspended in 12 M H_2SO_4 and refluxed in 0.358 M H_2SO_4 for 6 hrs) and analysed for neutral sugars (as alditol acetates by GC on an OV-225 column) and Klason-lignin according to Bethge et al. (1971). The identity of the sugars were confirmed with GC-MS (Lönngren and Svensson, 1974) and by paper chromatography (system b) on the hydrolysates.

Supernatant C and dialyzed supernatant C were cation exchanged (Dowex 50, H^+) and lyophilized. Samples containing 10 mg of these materials and 1 mg *myo*-inositol (internal standard) were hydrolyzed (0.25 M H_2SO_4 on boiling water bath for 16 hrs), neutralized ($BaCO_3$) and analyzed for neutral sugars as alditol acetates according to Sawardecker et al. (1965). The identity

of the sugars were confirmed by paper chromatography (system b) on the hydrolysates.

A part of supernatant C was deionized (Dowex 50, H^+ and Amberlite IR-4B, free base) and lyophilized. A 10.0 mg sample of this material containing 1.00 mg myo-inositol (internal standard) was silylated as described by Sweely et al. (1963). Low-molecular weight carbohydrates were then determined by GC as described by Theander and Åman (1976). The identity of the sugars was confirmed by paper chromatography (system a) on the deionized supernatant C.

Uronic acids. Samples of the isolates (0.5 g) and of lyophilized supernatant C (0.5 g) were deproteinized with hot aqueous phenol according to Westphal et al. (1965). The aqueous phase was dialyzed and the remaining carbohydrates were lyophilized. After hydrolysis (0.25 M H_2SO_4 on boiling water bath for 16 hrs), neutralization ($BaCO_3$) and treatment with a cation exchange resin (Dowex 50, H^+), the uronic acids were analyzed qualitatively by paper chromatography (system a) and paper electrophoresis. Uronic acids in the meal residue were analyzed in the same way, except that the deproteinizing step was omitted and the more severe hydrolysis was used (suspension in 12 M H_2SO_4 and refluxing in 0.358 M H_2SO_4 for 6 hrs).

RESULTS

From Table 1 and from the data given i Figure 1 it can be calculated that the rapeseed meal contained about 20% neutral sugars. When isolate C was prepared 54% of the neutral sugars of the meal was recovered in the meal residue and 43% in the supernatant.

As shown in Table 1, the isolates contained 2.3 - 2.8% of neutral sugars and 14.3 - 15.2% N after acid hydrolysis. Paper chromatography and paper electrophoresis showed that galactouronic acid was present after acid hydrolysis in the three isolates, and that no uronic acids could be detected in supernatant C. Thus, when preparing isolate C, all acidic carbohydrates in the alkaline rapeseed meal extract were accumulated in the isolate. Polysaccharides belonging to the pectin group, containing mainly arabinose, galactose, rhamnose and galactouronic acid, and amyloids, containing mainly glucose, xylose and fucose, seem to be the dominating carbohydrates (apart from CMC) in the isolates. No Klason-lignin could be detected in the isolates and their glucosinolate contents were below 0.003%.

The ribose present in the hydrolysate of the isolates is likely to derive from RNA. The RNA content was calculated from its ribose content assuming that the four RNA bases were present in equal amounts. This calculation (Table 1) showed that isolate A and C had high RNA contents (2.04 and 0.82% respectively) while that of isolate B was relatively low (0.16%). No ri-

bose could be detected in the supernatant C or in the meal residue. Thus, the RNA present in the meal was accumulated in the isolate during the rapeseed protein isolate process.

In the hydrolysate of isolate B, glucose was the dominating sugar. When this isolate was prepared CMC was added as a precipitation aid. In a previous study (Lönnerdal et al., 1976) it was shown that the added CMC was recovered in the isolate. Sugar analysis of hydrolysates of the CMC showed that the CMC contained 13.4% unsubstituted glucose. If it is assumed that the neutral sugars (except ribose) deriving from the rapeseed meal present in isolate B are composed of equal amounts of glucose and the other sugars analysed, the content of CMC in isolate B can be calculated as 12.4%. If all the glucose in the isolate originated from CMC the isolate would have contained 15.3% CMC.

When extracting the rapeseed meal, the extraction conditions were choosen such as to give a low amount of dissolved phytic acid (Gillberg and Törnell, 1976 a). This explains why the isolates had a low (< 0.32%) and the meal residue a high (5.2%) content of phytic acid. Separate experiments have shown that rapeseed protein isolates will contain substartial amounts of phytic acid if other extraction procedures are used in which phytic acid from the meal enters solution.

The meal residue contained 12% Klason-lignin which is built up of polyphenolic compounds and some carbohydrates and proteins. The Klason-lignin is mainly derived from the seed hulls present in the meal. Sugar analysis on hydrolysates of the meal residue showed that it contained 39.2% neutral sugars of which the dominating ones were glucose, arabinose, xylose and galactose (Table 1). Galactouronic acid and small amounts of glucouronic acid were also found to be present. These results indicate that cellulose, amyloids and acidic arabinogalactans were the dominating carbohydrates present in the meal residue.

Sugar analysis of the hydrolysed supernatant C (Table 1) showed that 32.5% of the dry material consisted of neutral sugar. Since most of the fructose is destroyed during the acid hydrolysis the real carbohydrate content must be higher. Of the dissolved substances in supernatant C, 25.5% was sucrose, 3.1% stachyose and 1.1% raffinose. Small amounts of fructose, glucose, digalactosylglycerol and galactinol were also present. Thus, about 90% of the carbohydrates in the supernatant were analysed as oligosaccharides. After dialysis, 23% of the dry matter content of supernatant C remained. Sugar analysis (Table 1) showed that 13.6% of the material consisted of neutral sugars with arabinose (67%) and galactose (22%) as the dominating ones. As no uronic acids could be detected in the hydrolysates this indicates that the dominating polysaccharides were arabinans and arabinogalactans.

DISCUSSION

The fact that when preparing isolate C the acidic carbohydrates and the RNA present in the alkaline rapeseed meal extract were completely recovered in the isolate, undoubtedly was a result of the strong tendency of polyacids to form insoluble complexes with proteins at neutral and acidic pH:s. The formation of insoluble complexes of this type has been observed in numerous systems (Bungenberg de Jong, 1949; Morawetz and Hughes, 1952; Nitschmann et al., 1959; Spinelli and Koury, 1970; Gillberg and Törnell, 1976 b). There can also be no doubt that in the formation of protein isolates from other raw materials than rapeseed that interactions are important between proteins and polyacids such as phytic acid, RNA, acidic carbohydrates and acidic polyphenols. Not only the proteins but also the polyacids extracted from the raw material will tend to accumulate in the protein isolates.

REFERENCES

Aspinall, G.O. and Jiang, K.-S. 1974. Rapeseed hull pectin. Carbohyd. Res. 38:247.

Bethge, P.O., Rådeström, R. and Theander, O. 1971. Kvantitativ kolhydratbestämning - en detaljstudie. Meddelande från STFI 63B.

Bungenberg de Jong, H.G. 1949. Complex colloid systems. In "Colloid science", Ed. Kruyt, H.R. Elsevier Publishing Company, The Netherlands.

Gillberg, L. and Törnell, B. 1976 a. Preparation of rapeseed protein isolates. I. Dissolution and precipitation behaviour of rapeseed proteins. J. Food Sci. In press.

Gillberg, L. and Törnell, B. 1976 b. Preparation of rapeseed protein isolates. II. Precipitation of rapeseed proteins in the presence of polyacids. J. Food Sci. In press.

Larm, O., Theander, O. and Åman, P. 1975. Structural studies on a water-soluble arabinan isolated from rapeseed (*Brassica napus*). Acta Chem. Scand. B. 29:1011.

Larm, O., Theander, O. and Åman, P. 1976. Structural studies on a water-soluble arabinogalactan isolated from rapeseed (*Brassica napus*). Acta Chem. Scand. B. In press.

Lönnerdal, B., Gillberg, L. and Törnell, B. 1976. Preparation of rapeseed protein isolates. III. A study of rapeseed protein isolates by molecular sieve chromatography. To be published.

Lönngren, J. and Svensson, S. 1974. Mass spectrometry in structural analysis of neutral carbohydrates. Advan. Carbohyd. Chem. Biochem. 24:41.

Morawetz, H. and Hughes, W.L. Jr. 1952. The interaction of proteins with synthetic polyelectrolytes. I. Complexing of bovine serum albumin. J. Phys. Chem. 56:64.

Nitschmann, von Hs., Rickli, E. and Kistler, P. 1959. "Ueber dem Einfluss der Polyphosphate auf die Löslichkeit einiger Plasmaproteine und die Möglichkeit der Plasma-Fraktionierung mit Polyphosphat". Helv. Chim. Acta 42:2198.

Persson, S. 1974. A method for determination of glucosinolates in rapeseed as TMS-derivatives. Proceedings. 4. Internationaler Rapskongress, Giessen, Germany, p. 381.

Sawardeker, J.S., Sloneher, J.H. and Jeans, S. 1965. Quantitative determination of monosaccharides as their alditol acetates by gas liquid chromatography. Anal. Chem. 37:1602.

Siddiqui, I.R. and Wood, P.J. 1971. Structural investigation of water-soluble rapeseed (Brassica campestris) polysaccharides. Part I. Rapeseed amyloid. Carbohyd. Res. 17:97.

Siddiqui, I.R. and Wood, P.J. 1972. Structural investigation of water-soluble rapeseed (Brassica campestris) polysaccharides. Part II. An acidic arabinogalactan. Carbohyd. Res. 24:1.

Siddiqui, I.R. and Wood, P.J. 1974. Structural investigation of oxalate-soluble rapeseed (Brassica campestris) polysaccharides. Part III. An arabinan. Carbohyd. Res. 36:35.

Siddiqui, I.R., Wood, P.J. and Khanzada, G. 1973. Low molecular weight carbohydrates from rapeseed (Brassica campestris) meal. J. Sci. Food Agric. 24:1427.

Spinelli, J. and Koury, B. 1970. Phosphate complexes of soluble fish proteins. Their formation and possible uses. J. Agr. Food Chem. 18(2):284.

Sweeley, C.C., Bentley, R., Makita, M. and Wells, W.W. 1963. Gas-liquid chromatography of trimethylsilyl derivatives of sugars and related substances. J. Am. Chem. Soc. 85:2497.

Theander, O. and Åman, P. 1974. Carbohydrates in rapeseed and turnip rapeseed meals. Proceedings. 4. Internationaler Rapskongress, Giessen, Germany, p. 429.

Theander, O. and Åman, P. 1976 a. Low-molecular carbohydrates in rapeseed and turnip rapeseed meals. Swed. J. Agric. Res. In press.

Theander, O. and Åman, P. 1976 b. Unpublished results.

Theander, O., Åman, P., Miksche, G. and Yasuda, S. 1976. Carbohydrates, lignin and polyphenols in turnip rapeseed and white mustard hulls of different colour. To be published.

Westphal, O. and Jaun, K. 1965. Bacterial Lipopolysaccharides. Extraction with phenol-water and further applications of the procedure. Methods Carbohyd. Chem. 5:83.

Wheeler, E.L. and Ferrel, R.E. 1971. A method for phytic acid determination in wheat and wheat fraction. Cereal Chem. 48:312.

ACKNOWLEDGEMENTS

We are indepted to Professor Olof Theander and Professor Bertil Törnell for valuable discussions, to Mrs Kristina Holmgren for skilled technical assistance, to Dr Klas Anjou for the glucosinolate analyses and to the Swedish Board for Technical Development for financial support.

Table 1. Composition of the products analyzed.

Component	Isolate A	Isolate B	Isolate C	Supernatant C	Dialyzed Supernatant C	Meal residue
Rhamnose [a]	1.3	tr	1.0	1.4	tr	2.5
Fucose [a]	1.7	-	1.1	-	tr	1.6
Ribose [a]	34.8	2.9	16.7	-	-	-
Arabinose [a]	19.9	8.0	17.0	8.6	66.5	34.7
Xylose [a]	2.5	2.1	2.2	0.8	5.5	11.3
Mannose [a]	10.5	3.2	8.3	4.7	tr	2.2
Galactose [a]	10.5	1.9	6.3	14.2	21.7	8.4
Glucose [a]	18.8	81.9	47.5	70.4	6.7	39.2
Neutral sugars [b]	2.8	2.5	2.3	32.5	13.6	21.9
N [b]	15.2	14.3	14.5	4.1	c	4.9
Phytic acid [b]	0.00	< 0.32	< 0.14	c	c	5.2
RNA [b]	2.04	0.16	0.82	-	-	-

a) relative composition of neutral sugars %

b) The figures are given as weight % of the materials on an oven-dry basis

c) not determined

Text to the figures

Figure 1. Scheme for the preparation of the isolates. The figures represent the approximate amounts (g) of dry substance produced from 100 g rapeseed meal.

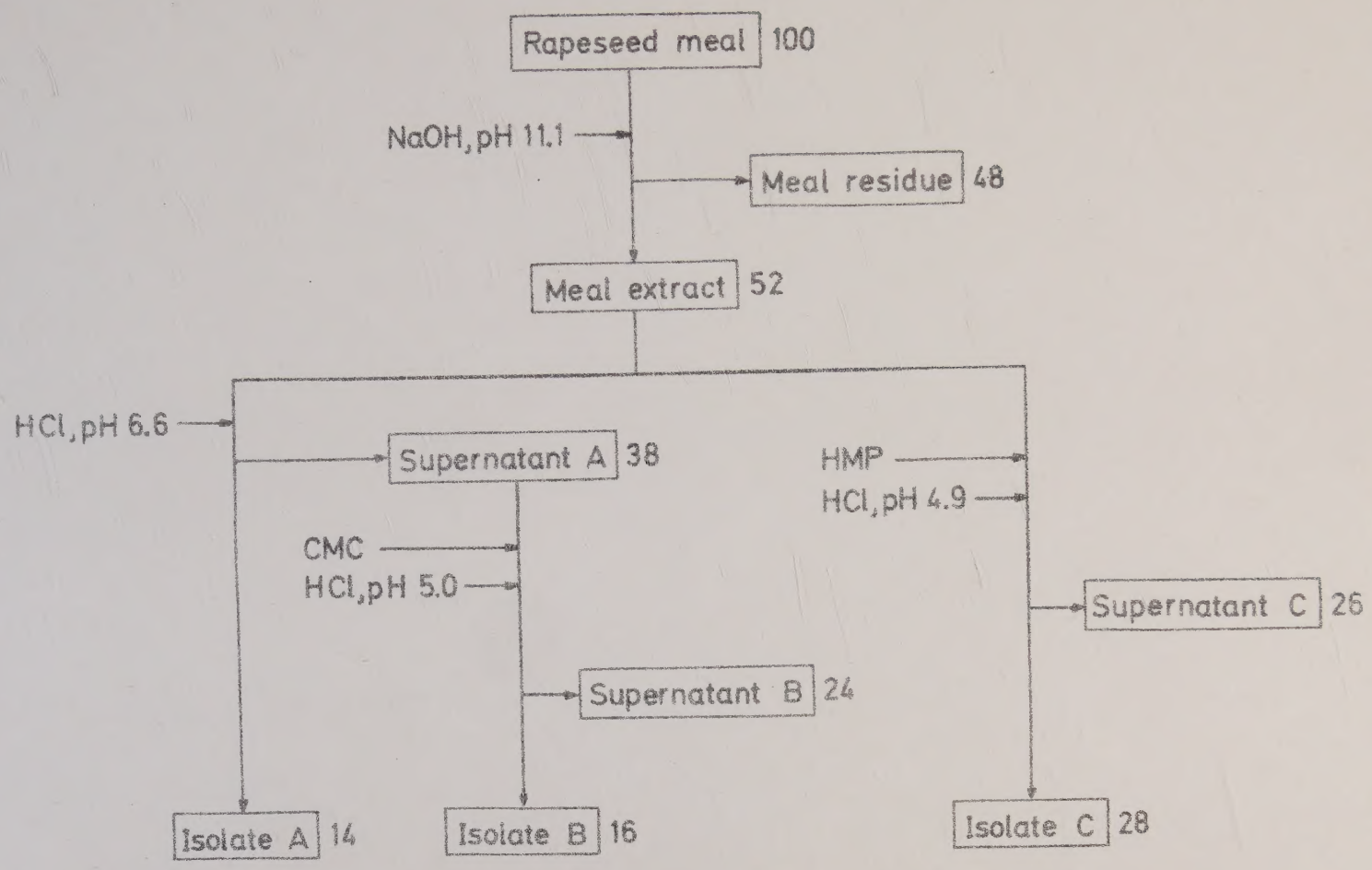

Rapeseed meal
100
NaOH, pH 11.1
Meal residue
48
Meal extract
52
HCl, pH 6.6
Supernatant A
38
HMP
HCl, pH 4.9
CMC
HCl, pH 5.0
Supernatant C
26
Supernatant B
24
Isolate A
14
Isolate B
16
Isolate C
28

FIGURE 1